

Epithelial remodelling of the conjunctiva in ocular allergy

A Thesis submitted for the degree of the Doctor of Medicine (by Research)

Nikolaos D. Georgakarakos

Institute of Ophthalmology,
University College London,
London UK

and

Moorfields Eye Hospital NHS Foundation Trust,
City Road, London UK.

ABSTRACT

Background: The spectrum of ocular allergy includes reversible conditions (seasonal, perennial and giant papillary conjunctivitis) and irreversible chronic severe forms of disease involving the cornea (vernal and atopic keratoconjunctivitis). Conjunctival remodelling may play a role in the pathogenesis of chronic allergic eye disease (CAED). Based on findings on asthmatic airway epithelia, it is suggested in chronic asthma that activation of the epithelial-mesenchymal trophic unit (EMTU hypothesis) and EGFR may participate in disease development and account for chronicity, severity and poor response to steroid treatment. This thesis investigates the potential application of the EMTU hypothesis to CAED.

Aims and Methods: Conjunctival biopsies of healthy subjects, SAC, GPC (controls group) were compared to VKC and AKC (CAED group) and conjunctival tissues of active and treated OCP. Expression of EGFR, TGF α and CD44 was assessed by means of immunohistochemistry. Cells of two human epithelial conjunctival cell lines (IOBA-NHC & CHwK) received single and multiple cytokine treatments (PMA, IL-17A, TNF α /IL-1 β) and secretion of EGFR, VEGF, CD44, TGF α and p21^{waf} of harvested supernatants were assessed by means of ELISA. Statistical analysis was performed using Mann-Whitney test for the immunohistochemistry and t-test for the ELISA results.

Results: Immunohistochemistry studies revealed that there was increased expression of all EMTU remodelling markers in the CAED group compared to Controls ($p < 0.01$). OCP conjunctival biopsies showed no expression for EGFR and TGF α . There was increased secretion of the remodelling molecules by both cell lines after single/multiple cytokine treatments.

Conclusions: Epithelial conjunctival remodelling may be responsible for disease severity in CAED as shown in chronic asthma. Epithelial cell EGFR-mediated remodelling parallels the observations made in chronic asthmatic epithelia and the results are in agreement with previous studies in biopsies and tears of VKC patients. Finally suggestions for new treatment strategies are proposed to prevent or inhibit disease perpetuation.

DECLARATION

The work presented in this thesis is my own and where information has been derived from other sources, I confirm that this has been indicated below:

Ocular allergy biopsies used in this thesis were obtained from a tissue bank maintained by our group from previous research work by Dr Melanie Hingorani. OCP biopsies were kindly provided by Dr Valerie Saw and Prof John Dart. Immunohistochemistry principles and techniques were adapted from Dr Daniela Metz's PhD thesis.

All experiments were designed, developed and performed by myself. Additionally, the sCD44 ELISA assay of the pilot *in vitro* studies and the FACS analysis of the cell viability study were performed by Miss I. Offiah. VEGF/EGFR ELISA assays of steroid treated cell lines were performed by Mr J. Porter.

Mr Nikolaos D. Georgakarakos

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CONTENTS**PAGE****Abbreviations**

17-18

CHAPTER 1: INTRODUCTION**The role of the epithelium in inflammation and remodelling in asthma and ocular allergy**

1.1	Overview	20
1.2	The epithelium: cell biology	22
1.2.1	Epithelial classification	23
1.2.2	Cell biology of the conjunctival epithelium	26
1.2.3	Epithelial integrity, cellular adhesion and barrier actions	29
1.3	Allergic inflammation of the airway and conjunctival mucosa	30
1.3.1	T cell derived cytokines and allergic asthma	31
1.3.2	The roles of fibroblasts	32
1.4	Tissue remodelling	34
1.4.1	Growth factors and soluble mediators in tissue remodelling	36
1.4.2	Eosinophils and remodelling	37
1.4.3	The roles of Matrix Metalloproteinases in remodelling	37
1.5	Asthma and the epithelial mesenchymal trophic unit (EMTU) hypothesis	39
1.5.1	Chronic asthma: altered response to injury and epithelial mesenchymal communication	40
1.5.2	Th1 and Th2 participation: essential but not enough to account for disease severity	42
1.5.3	Mechanisms of epithelial damage in asthma: epithelial mesenchymal communication	44
1.5.3.1	The role of growth factors and the EMTU hypothesis	44
1.5.3.2	The role of CD44 in enhancing EGFR signalling	46
1.5.3.3	The roles of transforming growth factor- β and the TGF β -EGF axis.	48
1.5.3.4	Regulation of epithelial cell population: The role of p21 cyclin dependent kinase inhibitor	50
1.5.3.5	EGFR and growth factors in chronic allergic eye disease	51

1.6	The role of the conjunctival epithelium in ocular allergy	53
1.6.1	Cellular participation and cytokines	53
1.6.2	The pro-inflammatory roles and antigen presentation capabilities of the conjunctival epithelium	54
1.6.3	The roles of eosinophils in ocular allergy	57
1.6.4	The roles of T cell subsets	58
1.7	The immunopathogenesis of ocular allergy	61
1.7.1	The roles of T cells	61
1.7.2	Pathogenesis of IgE-mediated diseases: seasonal and perennial allergic conjunctivitis	63
1.7.3	Current understanding of the immunopathogenesis of atopic and vernal keratoconjunctivitis	65
1.7.4	Giant papillary conjunctivitis: Non IgE mediated allergy	67
1.8	Classification of Ocular Allergic diseases	69
1.9	Clinical characteristics and forms of ocular allergy	71
1.9.1	Seasonal and perennial allergic conjunctivitis (SAC & PAC)	71
1.9.2	Vernal Keratoconjunctivitis (VKC)	72
1.9.3	Atopic Keratoconjunctivitis (AKC)	74
1.9.4	Giant Papillary Conjunctivitis (GPC)	76
1.9.5	Treatment strategies in ocular allergy	77
1.9.6	Chronic autoimmune conjunctival inflammatory disease: ocular cicatricial pemphigoid.	79
1.10	Summary	80
1.11	Aims and Hypothesis	82

CHAPTER 2: MATERIALS & TECHNIQUES

2.1	Conjunctival biopsies	85
2.1.1	Biopsy of non-inflamed subjects	85
2.1.2	SAC biopsies	85
2.1.3	Biopsy of patients with GPC, VKC and AKC	86
2.1.4	Biopsies from OCP patients	86
2.1.5	Antibody optimisation studies	87
2.1.6	Ocular allergy and OCP groups participating in the study	89
2.1.7	Immunohistochemistry principles and techniques	90
2.1.8	Preparation of biopsies	91
2.1.9	Single antibody immunohistochemistry	92
2.1.10	Immunostaining: grading and analysis	93
2.2	<i>In vitro</i> studies	95
2.2.1	Conjunctival epithelial cell lines	95
2.2.1.1	<i>In vitro</i> culture of IOBA-NHC line	95
2.2.1.2	Defrosting cells	96
2.2.1.3	Passaging cells	96
2.2.1.4	Cell density calculations	97
2.2.1.5	<i>In vitro</i> culture of ChWK line	98
2.2.2	Cytokine treatments of epithelial conjunctival cell lines	98
2.2.3	Pilot experiments using IOBA-NHC line with single cytokine treatment	100
2.2.4	Single cytokine treatments of IOBA-NHC cells	101
2.2.5	Multiple cytokine treatments of IOBA-NHC cells	101
2.2.6	IOBA-NHC with steroid treatment and the effect of IL-17E (IL-25)	102
2.2.7	Multiple treatments of the ChWK line	102
2.3	Enzyme-linked immunosorbent assay (ELISA) technique	103
2.3.1	Calculation of standard curves	105
2.3.2	Statistical analysis	105
2.4	Epithelial cell viability studies	106
2.4.1	Apoptosis assay	108

CHAPTER 3: RESULTS- IMMUNOHISTOCHEMISTRY STUDIES

Conjunctival epithelial remodelling in CAED and OCP: Expression of epithelial-mesenchymal communication associated molecules.

3.1	Introduction	110
3.2	Preliminary antibody optimisation immunohistochemistry results	110
3.3	Antibody expression results	114
3.3.1	EGFR immunostaining (1:200)	114
3.3.2	TGF α immunostaining (1:100)	115
3.3.3	CD44 immunostaining (1:200)	116
3.3.4	Summary of immunostaining data of ocular allergy biopsies	117
3.3.5	Immunostaining results of OCP biopsies	121
3.4	Discussion of results	122
3.4.1	Expression of epithelial remodelling markers in the controls group	122
3.4.2	Expression of epithelial remodelling markers in CAED group (AKC/VKC)	123
3.5	Summary	128

CHAPTER 4: RESULTS - *IN VITRO* STUDIES

**Responses to inflammation-related molecules by conjunctival epithelial cells:
An *in vitro* model of ocular inflammation and abnormal repair.**

4.1	Introduction	130
4.2	IOBA- NHC and CHwK cell lines: properties and experimental uses	133
4.2.1	Morphologic and functional characterisation	133
4.2.2	Cytokines response profile	134
4.2.3	Muscarinic and adrenergic receptors	134
4.2.4	ChWK cell lines: Morphological and functional characterisation	135
4.2.5	Immunostimulatory properties of CHwK cells	135
4.3	Standard curves of analytes with examples	136
4.4	Results of <i>in vitro</i> pilot study with IOBA-NHC line after a single initial treatment and supernatant harvest at 24, 48, 76, 92 hours	137
4.4.1	EGFR secretion	137
4.4.2	VEGF secretion	139
4.4.3	sCD44 secretion	141
4.4.4	TGF α secretion	143
4.4.5	p21 secretion	143
4.5	Pilot <i>in vitro</i> studies: sCD44 secretion by IOBA-NHC cells	144
4.5.1	sCD44 levels at 24, 48, 72, 96, 144, 192 hours in response to a single cytokine treatment.	144
4.5.2	Pilot <i>in vitro</i> studies to investigate sCD44 secretion at 144 and 192 hours by IOBA-NHC cells after the use of combination of treatments	148
4.6	Yo-pro epithelial cell viability studies: apoptosis assay results	150
4.7	<i>In vitro</i> studies using a single treatment in IOBA-NHC cell lines	153
4.7.1	sCD44 secretion	153
4.7.2	EGFR secretion	154
4.7.3	TGF α secretion	155
4.7.4	Summary of findings	156
4.8	<i>In vitro</i> studies using multiple treatments in IOBA-NHC cell lines	157

4.8.1	EGFR secretion	157
4.8.2	sCD44 secretion	158
4.8.3	VEGF-A secretion	159
4.8.4	Summary of findings	160
4.9	<i>In vitro</i> studies using IOBA-NHC cells to investigate p21^{waf} levels after single treatment with PMA, IL-17A and TNFα/IL-1β at 144 and 192 hours.	161
4.10	ChWK cell line: EGFR and sCD44 secretion after multiple treatments and comparison with the IOBA-NHC line.	162
4.10.1	sCD44 secretion	162
4.10.2	EGFR secretion	163
4.10.3	p21 ^{waf}	164
4.11	Effects of steroid dexamethasone pre-treatment and IL-25 to the secretion of EGFR and VEGF by IOBA-NHC cells.	165
4.12	Discussion	169
4.12.1	IOBA-NHC and ChWK conjunctival epithelial cell lines express EMTU associated molecules after treatment with pro-inflammatory cytokines and IL-17	169
4.12.2	Advantages and limitations of IOBIA-NHC and ChWK lines in experimental use	171

CHAPTER 5: GENERAL DISCUSSION

5.1	Conjunctival epithelial remodelling and the application of EMTU hypothesis to CAED	175
5.1.1	EGFR and its ligand TGF α expression parallels disease severity in CAED	175
5.1.2	Proinflammatory cytokines TNF α /IL-1 β promote <i>in vitro</i> of EGFR and TGF α in conjunctival epithelial cell lines	177
5.1.3	EGFR and TGF α levels parallel eosinophilic activation and neutrophilia	178
5.1.4	Increased CD44 levels in CAED parallel disease severity	179
5.1.5	Increased p21 ^{waf} production <i>in vitro</i> by IOBA-NHC conjunctival epithelial cell parallels EGFR and CD44 profiles	180
5.1.6	TNF α /IL-1 β and IL-17A may induce VEGF secretion by conjunctival epithelial cells <i>in vitro</i>	182
5.1.7	Epithelial mediated remodelling in asthma and CAED	183
5.1.8	EGFR and associated epithelial remodelling mediators in CAED are not expressed in OCP	187
5.1.9	EGFR expression is not affected by steroids in severe asthmatic mucosa.	189
5.1.10	Epithelial-mesenchymal transition (EMT): repair and fibrosis	190
5.1.11	Summary and proposals originating from this thesis	191

5.2	Th17 T cell-derived cytokines cells may have roles in epithelial conjunctival remodelling in CAED	193
5.2.1	IL-17 induces EGFR secretion <i>in vitro</i> by human conjunctival epithelial cells	193
5.2.2	IL-25 (IL-17E) induces VEGF secretion <i>in vitro</i> by conjunctival epithelial cells with or without dexamethasone pre-treatment.	194
5.2.3	Toll like receptors and conjunctival epithelial cells	197

5.3.	Reclassification of ocular allergic disease and novel therapeutic strategies	199
5.3.1	Re-classifying ocular allergic disease	199
5.3.2	New treatments, strategies and further studies	202
5.3.3	EGFR inhibition as a possible new therapeutic strategy to control inflammation in chronic asthma and chronic allergic eye disease	204

REFERENCES	206 – 239
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APPENDIX – patient details	240 – 244
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LIST OF FIGURES

Figures	Page
----------------	-------------

CHAPTER 1

1.1	Diagrams showing structures of various types of epithelia	24
1.2	The surface of tracheal epithelium (SEM)	25
1.3	Micrograph of the primary bronchial epithelium	25
1.4	Forniceal conjunctival epithelium (TEM)	26
1.5	Conjunctival epithelial surface variation with age (SEM)	27
1.6	Histology slide of normal conjunctival tissue section	28
1.7	Activation of the EMTU in chronic asthma	41
1.8	Th2 –EMTU interactions in asthma pathogenesis	43
1.9	The role of CD44 enhancing EGFR mediated signalling	47
1.10	TGF β signalling via Smads and gene transcription	48
1.11	Immunostaining showing EGFR expression in VKC	51
1.12	Immunostaining showing VEGF expression in VKC	51
1.13	Effects of IL-17 on various types of cells	59
1.14	Clinical photograph of seasonal allergic conjunctivitis	71
1.15	Clinical photograph of papillae in VKC	72
1.16	Clinical photograph of the upper tarsal conjunctiva in GPC	76

CHAPTER 2

2.1	Micrograph showing negative control background staining of the epithelium	94
2.2	Example of mild (1+) antibody staining	94
2.3	Example of moderate (2+) antibody staining	94
2.4	Example of intense (3+) antibody staining	94
2.5	Illustration of ELISA method	104
2.6	Illustration of YO-PRO and PI method	106

CHAPTER 3

3.1	Indicative photomicrographs of CD44 antibody optimisation study	113
3.2	Photomicrographs of EGFR expression	114
3.3	Photomicrographs of TGF α expression	115
3.4	Photomicrographs of CD44 expression	116
3.5	Dot plot graphs of epithelial expression of antibodies	121
3.6	Dot plot graphs of stromal expression of antibodies	118
3.7	Dot plot graph of epithelial antibodies' expression (summary)	119
3.8	Dot plot graph of stromal antibodies' expression (summary)	119
3.9	Dot plot graph of epithelial and stromal antibodies' expression in the CAED group	120
3.10	Photomicrographs of EGFR epithelial expression in SAC, VKC, OCP	125
3.11	Photomicrographs showing TGF α expression in SAC, GPC and OCP.	125

CHAPTER 4

4.1	Examples of standard curves of all proteins assayed by ELISA	136
4.2	Pilot IOBA-NHC study: ERGR results for each time point	137
4.3	Pilot IOBA-NHC study: EGFR results for each treatment	138
4.4	Pilot IOBA-NHC study: VEGF results for each time point	139
4.5	Pilot IOBA-NHC study: VEGF results for each treatment	140
4.6	Pilot IOBA-NHC study: sCD44 results for each time point	141
4.7	Pilot IOBA-NHC study: sCD44 results for each treatment	142
4.8	Pilot IOBA-NHC study: sCD44 results for each time point until 192 hours	144
4.9	Pilot IOBA-NHC study: sCD44 results compared to untreated cells (A) TNF α /IL-1 β (B) PMA and (C) IL-17A	146
4.10	Pilot IOBA-NHC study: sCD44 results compared to untreated cells (A) Poly:IC (B) LPS (C) Grass extract (D) Zymozan	147
4.11	sCD44 production by IOBA-NHC cells using combinations of treatments (144 hours)	148
4.12	sCD44 production by IOBA-NHC cells using combinations of treatments (192 hours)	149

4.13	Flow cytometric analysis: epithelial cell viability assessment for the untreated cells.	151
4.14	Flow cytometric analysis YoPro-PI staining in conjunctival epithelial cells after 192 hours of cytokine treatment.	152
4.15	sCD44 levels at 24, 48, 72, 96 hours by IOBA-NHC cells.	153
4.16	EGFR levels at 24, 48, 72, 96 hours by IOBA-NHC cells.	154
4.17	TGF α secretion at 24, 48, 72, 96 hours by IOBA-NHC cells.	155
4.18	EGFR secretion after three treatments at various time by IOBA-NHC cells	157
4.19	sCD44 secretion after three treatments at various timepoints by IOBA-NHC cells.	158
4.20	VEGF production after three treatments (192 hours) by IOBA-NHC cells.	159
4.21	Increased p21 ^{waf} production after treatment with PMA, TNF α /IL-1 β and IL-17A (144 & 196 hours).	161
4.22	sCD44 levels by ChWK & IOBA-NHC lines after three treatments (196 hours)	162
4.23	EGFR levels by IOBA-NHC and CHwK conjunctival epithelial cells after treatment with PMA, TNF α /IL-1 β , IL-17 (192 hours).	163
4.24	p21 levels by CHwK cells after treatment with PMA, TNF α /IL-1 β , IL-17 (144 & 192 hours).	164
4.25	VEGF secretion after dexamethasone pre-treatment by IOBA-NHC cells after treatment with PMA, TNF α /IL-1 β , IL-17 (48 hours).	167
4.26	VEGF and EGFR secretion with and without dexamethasone pre-treatment by IOBA-NHC cells after treatment with PMA, TNF α /IL-1 β , IL-17, IL-25 (48 hours).	168

CHAPTER 5

5.1	A proposal of the application of EMTU hypothesis in CAED based on the results of this thesis.	191
5.2	A proposal for the interactions between proinflammatory/Th17 mediated inflammation and the EMTU in CAED pathogenesis.	196
5.3	The New classification for ocular allergy (EAACI Task Force, 2012).	200
5.4	Multiple targets of immunomodulation in allergic disease.	202

LIST OF TABLES

Table **Page**

CHAPTER 1

1.1	Features of airway remodelling	35
1.2	Overview of cells and mechanisms in ocular allergy	53
1.3	Tear mediators, cytokines and cells detected in active ocular allergies	55
1.4	Types of ocular allergic disease and their classification	70

CHAPTER 2

2.1	Antibodies used for optimisation study	88
2.2	Final antibody dilutions used in the study	89
2.3	Grading system for biopsy sections	93
2.4	Recombinant cytokine dilutions used to treat cells	99
2.5	Recombinant cytokine preparation technique	100
2.6	Proteins of interest targeted for detection by ELISA	104

CHAPTER 3

3.1	Preliminary optimisation antibody data – epithelial expression	111
3.2	Preliminary optimisation antibody data – stromal expression	112
3.3	Antibody grading scores of antibodies in OCP	121

CHAPTER 4

4.1	t- tests for CD44 levels of IOBA-NHC epithelial cells treated with PMA, TNF α /IL-1 β , IL-17 compared to untreated cells.	153
4.2	t- tests for TGF α levels of IOBA-NHC epithelial cells treated with PMA, TNF α /IL-1 β , IL-17 compared to untreated cells.	155
4.3	Summary of responses of the remodelling markers after a single cytokine treatment.	156
4.4	t- tests for VEGF levels of IOBA-NHC epithelial cells treated with PMA, TNF α /IL-1 β compared to untreated cells.	159
4.5	Summary of the secretion of remodelling molecules in response to multiple cytokine treatments by IOBA-NHC cells	160
4.6	Summary of the effects of different cytokines to p21 secretion by two conjunctival cell lines.	164
4.7	Summary of the secretion of VEGF in response to various cytokine treatments with and without dexamethasone pre-treatment.	166

CHAPTER 5

5.1	Table showing the cellular and cytokine participation in ocular allergy including the results of this Thesis.	192
5.2	A proposal for the features used for the classification of ocular allergy	201

Abbreviations

AKC	Atopic Keratoconjunctivitis
α -SMA	α - Smooth Muscle Actin
bFGF	basic Fibroblast Growth Factor
BMP-7	Bone Morphogenetic Protein -7
CAED	Chronic allergic eye disease
CDK	Cyclin Dependent Kinase
DC	Dendritic cells
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial Mesenchymal Transition
EMTU	Epithelial Mesenchymal Trophic Unit
FSP1	Fibroblast Specific Protein1
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GPC	Giant Papillary Conjunctivitis
IFN- γ	Interferone γ
LPS	Lipopolysaccharide
MMP	Matrix Metalloproteinases
OCP	Ocular Cicatricial Pemphigoid
PAC	Perennial Allergic Conjunctivitis
PCNA	Proliferating Cell Nuclear Antigen
PDGF	Platelet Derived Growth Factor
PMA	Phorbol myristate acetate
Poly I:C	Polyribonucleosinic polyribocytidylic acid
RANTES	Regulated upon activation, normal T cells expressed and secreted.

SAC	Seasonal Allergic Conjunctivitis
TGF- α	Transforming Growth Factor- α
TGF- β	Transforming Growth Factor- β
TNF α	Tumour Necrosis Factor α
TIMPs	Tissue Inhibitors of Matrix Metalloproteinases
TLR	Toll Like Receptor
TSLP	Thymic Stromal Lymphopoietin
VEGF	Vascular Endothelial Growth Factor
VKC	Vernal Keratoconjunctivitis

CHAPTER 1

INTRODUCTION

The role of the epithelium in inflammation and remodelling in asthma and ocular allergy

1.1 Overview

Allergic conjunctivitis is a group of inflammatory diseases of the ocular surface which can be acute or chronic, mild or severe and it includes acute disorders, seasonal (SAC) and perennial allergic conjunctivitis (PAC) as well as chronic or persistent forms of disease, vernal keratoconjunctivitis (VKC), atopic keratoconjunctivitis (AKC) and giant papillary conjunctivitis (GPC). Common to these diseases are a papillary conjunctivitis and a type I IgE mediated hypersensitivity reaction (except GPC), although each disease category has unique immunopathogenic features.

Seasonal and perennial allergic conjunctivitis represent the commonest forms of ocular allergy which are mild, resolve either spontaneously or due to treatment and are not sight threatening. VKC and AKC are rarer but more severe with corneal involvement which can cause sight impairment. GPC is usually seen in contact lens users and patients with ocular prosthetic implants. At the moment we accept that there is an overlap between the phenotypes of the severe forms of ocular allergy and rely on clinical features to make the diagnosis and decisions on treatment.

The conjunctiva being a mucosal membrane shares common structural and functional features with the mucosal lining of other systems such as the respiratory tract. It has been suggested by Prof Stephen Holgate and his team in Southampton, UK that an abnormal epithelial-mesenchymal communication (the EMTU hypothesis) may explain the disease severity in chronic asthma.

In the course of Chapter 1 an account is given of the basic known structural and functional properties of the epithelial cells as part of mucosal membranes. Hingorani's work on the central role of the epithelium in ocular allergy pathogenesis provides stronger insight and forms the basis for future work on its immunopathogenesis and treatment. It was suggested that it has pro-inflammatory roles in chronic allergic eye disease with involvement in leucocyte recruitment and cytokines upregulation promoting eosinophilia. Its role in allergic inflammation will be presented, focusing on allergic eye disease and asthma.

Prof Holgate's hypothesis on asthma pathogenesis will be presented along with a discussion about the role of remodelling markers. The EMTU hypothesis for the

pathogenesis of asthma will be adapted for application to allergic conjunctivitis. A claim of this study is that since the respiratory tract and conjunctiva are both mucosal membranes sharing common structural and functional characteristics, a similar hypothesis may apply to VKC and AKC. The role of remodelling in conjunctival inflammatory disease will be presented together with the concept of epithelial-mesenchymal interaction in mucosal inflammatory diseases based on asthma as proposed by Prof Stephen Holgate and his team.

Furthermore in Chapter 1, a detailed presentation of the clinical features and classification of ocular allergy is given highlighting the overlap of features and frequent similarities in clinical presentation as well as the need to achieve disease control. The study continues with an inquiry into the adequacy of the classification systems available to date.

1.2 Epithelial Cell biology

Epithelial cells form the major constituent of various tissues in the body lining the surface of organs or internal cavities and tracts and are arranged in cell sheets with the help of intercellular adhesive cell junctions. Basement membranes separate the epithelial layer from the underlying tissues and depend on metabolite and oxygen diffusion from supporting tissues. Therefore they are involved in a great variety of processes such as the regulation of secretion and adsorption in organs such as the skin, gut, and lungs, and the protection of the sub-epithelial tissue components from pathogenic microbes, toxic factors and physical trauma to which they are directly exposed.

In the lung the respiratory tract consists of an epithelial layer, basement membrane, lamina propria and cartilages. The epithelial layer is mainly composed of pseudostratified columnar ciliated epithelial cells and separated from the lamina propria by a basement membrane. The lamina propria contains fibrous tissue with blood and lymphatic vessels as well as smooth muscle cell fibres. The epithelium together with the basement membrane and the lamina propria form a mucosa.

The epithelium acts a physical barrier between the external environment and the respiratory system responsible for gas exchange. Additionally it has immunological and metabolic properties. In asthma it is known that epithelial damage causes increased permeability and easier access of antigens and allergens to the subepithelial components. In atopic conditions such as asthma and dermatitis the epithelium is dysregulated leading to increased permeability and access to airborne allergens (1-2).

1.2.1 Epithelium classification

Epithelia are classified based on the number of cell layers, the shape of the cells and the presence of associated features at the surface such as cilia or keratin. More specifically they can be a simple cell monolayer or stratified in two or more layers. The whole body has an epithelial covering, the skin. Furthermore the gastrointestinal tract, respiratory tract, urinary tract all have epithelial cell linings facing the lumen. Glandular and exocrine secretions occur via an epithelial duct. The conjunctiva as its original definition suggests, is a continuous mucosal translucent thin membrane joining the eyeball to the lids and, consists of a superficial epithelial layer and connective tissue stroma. It forms a mechanical barrier on surfaces exposed to the external environment but can be also specialized to perform specific functions such as removing inhaled debris with cilia in the respiratory tract, nutrients' absorption from the gastrointestinal tract, or mucin production and fluid for lubrication and transport in ducts.

Types of epithelia are described below and illustrated in Figure 1.1:

- a) Squamous epithelium: cells at the surface have smaller nuclei and greater amounts of keratin and form a multi-layered structure. If the mucosal membrane is involved in significant "wear and tear" it is covered by a thick layer of acellular keratin (e.g. skin), otherwise it is non-keratinizing squamous epithelium (e.g. cervix).
- b) Columnar epithelium: the cells are taller along a basal lamina, typically line glandular ducts, produce mucin and can be ciliated (e.g. colon).
- c) Pseudostratified columnar epithelium: in this type of epithelium not all the cells along the basal lamina reach the luminal surface, but all contact the basal lamina e.g. respiratory tract from the nasopharynx down into the bronchioles of the lung; ciliated. Illustrated in Figures 1.2 and 1.3.
- d) Cuboidal epithelium: the cells have a rounder shape and typically lines ducts draining glands and found in renal tubules. The small ducts of sweat glands (including breast, a modified sweat gland) can pile up to a stratified cuboidal appearance in larger ducts.

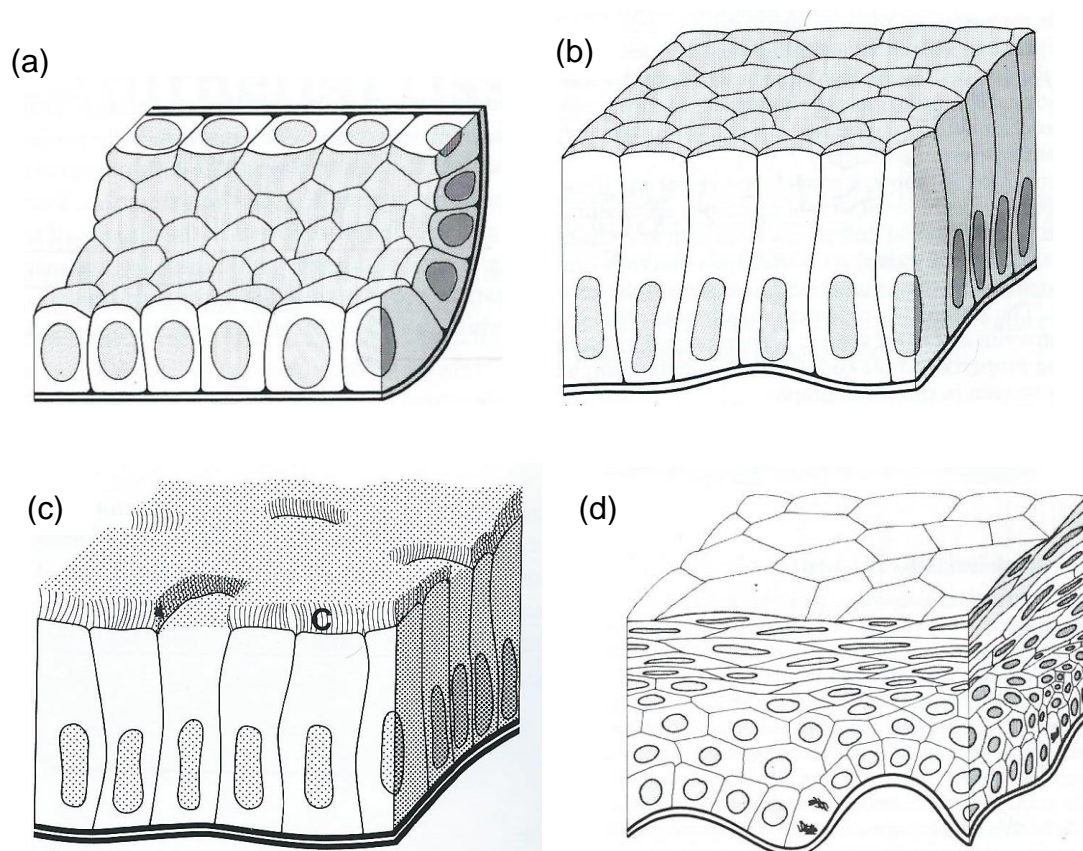


Figure 1.1: The structures of various types of epithelia. (a) simple cuboidal epithelium e.g. small collecting ducts in salivary glands, kidney, pancreas (b) simple columnar epithelium: typically found in highly absorptive surfaces (c) simple ciliated columnar epithelium: not common except in the female reproductive tract (d) stratified squamous epithelium: found on lining surfaces subject to mechanical abrasion and kept moist by glandular secretion (3).

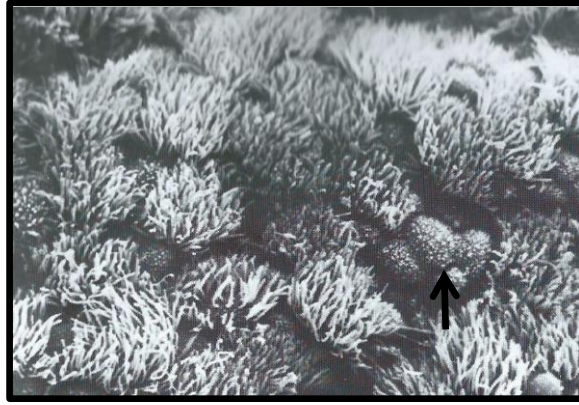
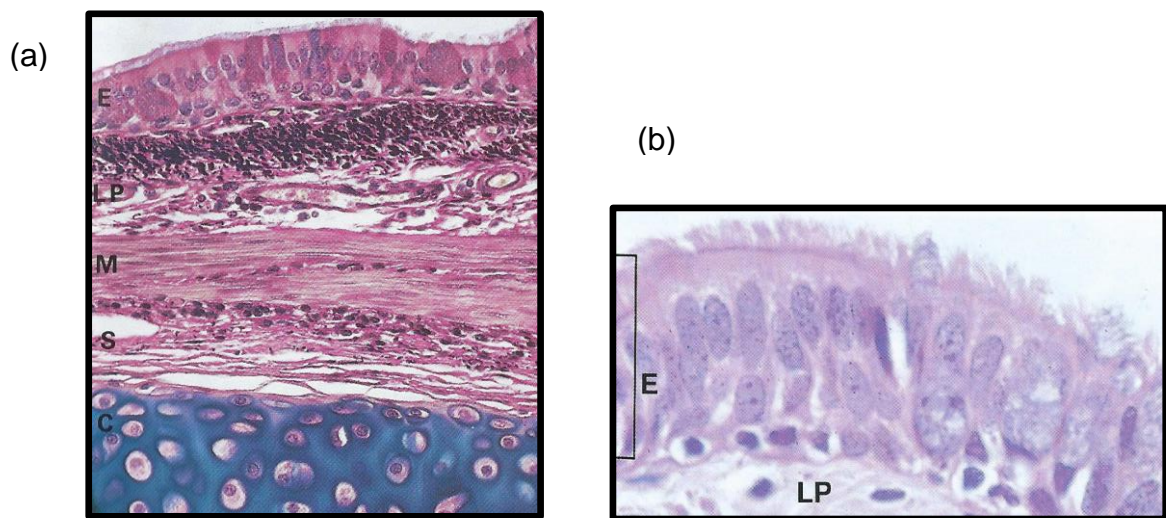


Figure 1.2: Scanning electron microscopy of the surface of the tracheal epithelium demonstrating the numerous cilia with goblet cells (black arrow); (magnification x2000) (4).



Figures 1.3 (a&b): Micrographs of the primary bronchus showing the epithelium (E) facing the lumen over the lamina propria (LP); Magnifications (a) x150 and (b) x900, (3).

The epithelium lies on a basement membrane that separates the epithelial cells from the underlying connective tissue stroma. Integrins in the epithelial cells extend into the basement membrane and attach to laminin which in turn attaches to type IV collagen and to fibronectin in the basal lamina. There are anchoring fibrils made of type VII collagen. Therefore the basal lamina acts as an anchoring point for the epithelium and forms a meshwork that acts as a barrier to trap or exclude molecules. The lamina reticularis of the basement membrane interfaces with the underlying connective tissue.

1.2.2 The conjunctival epithelium: cellular biology

The epithelial cells are attached with desmosomes across highly interdigitating borders and the basal region of the cells attaches to the basal lamina by hemidesmosomes via intermediate filaments (Figure 1.4). The cell density reduces with age (Figure 1.5). The superficial cells are joined by continuous anterior borders of junctional complexes that seal the intercellular spaces, which obstruct the passage of water soluble molecules and makes the conjunctiva a semipermeable membrane. The apical cytoplasm of the cells at the surface outermost layer contains numerous vesicles containing mucoprotein and is also known that their number is increased in allergic conditions.

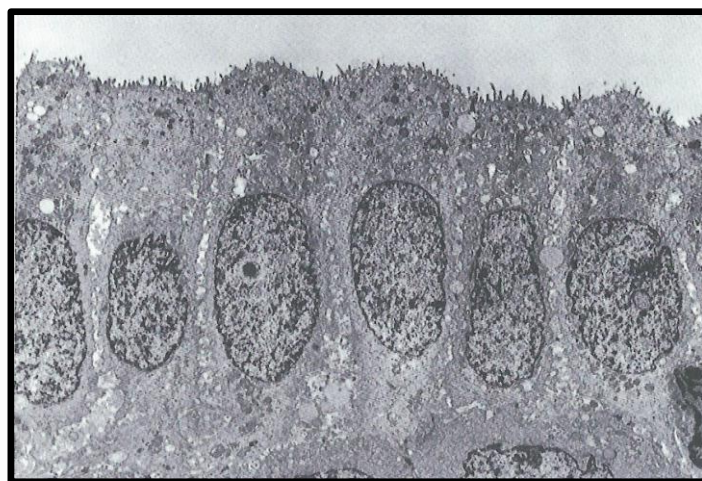
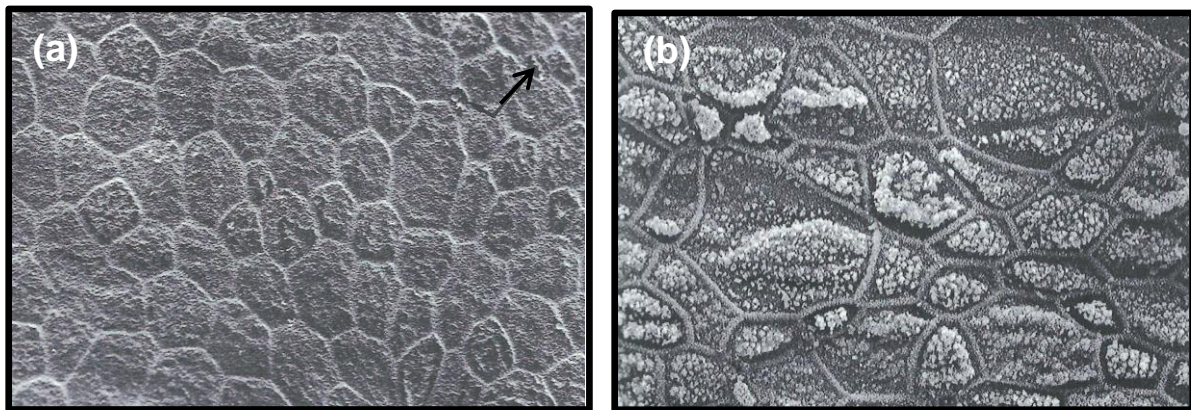


Figure 1.4: Transmission electron microscopy of fornical conjunctival epithelium in a 45 year old, showing columnar cells with apical microvilli (magnification x2500) (4).

Additionally throughout the conjunctival tissue the epithelium contains Goblet cells with highest population at the plica semilunaris at epithelial crypts most dense nasally. The goblet cells are the main cellular production of mucin which is a component of the tears moistening the ocular surface.



Figures 1.5: (a) scanning electron micrograph of the conjunctival epithelial surface from a 30 year old showing small polygonal cells and interspread goblet cells – black arrow (b) scanning electron micrograph of the conjunctival epithelial surface in a 77 year old showing pleomorphic cells and absence of goblet cells (4).

The conjunctiva being a semi-transparent mucosal membrane shares common features with the respiratory tract mucosa in health and asthmatic disease. It consists of an epithelial layer and a connective tissue stroma with a subepithelial mesenchymal cell layer, as shown in Figure 1.6 below.

In the embryo, it is known that the mesenchymal cells (also known as the embryonic fibroblasts) make the embryonic tissue and will differentiate later to fibroblasts, blood and blood-related organs (eg spleen). Some will remain in a “dormant” state below the epithelial layer and will be activated later in life, should the need arise e.g. tissue response to injury.

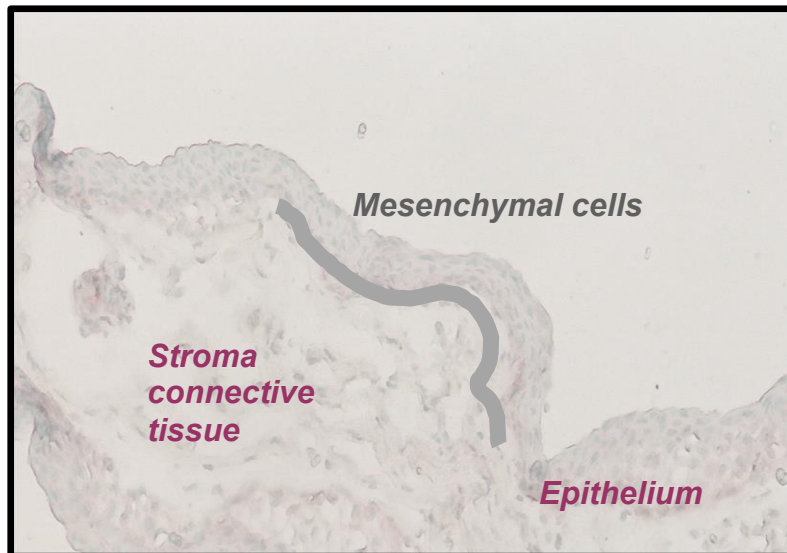


Figure 1.6: Histology of conjunctival tissue section showing the basic anatomical features of the mucosal membrane.

The mucosal membrane is well known to participate in the regulation of inflammation and leucocytes. More specifically the epithelium of the conjunctiva is thought to have a role in the pathogenesis of ocular allergy via cytokines and pro-inflammatory mediators as well as inflammatory cell regulation. Similar evidence exists for the respiratory mucosal epithelium in asthma.

This section aims to describe these roles and provide a detailed account of the evidence so far. Furthermore, the role of cytokines will be described and, finally the role of remodelling molecules.

1.2.3 Epithelial integrity, cellular adhesion and barrier actions

The integrity of the epithelium depends on the epithelial adhesion eg CD44, e-cadherin promoted by junctional and non-junctional cell adhesion molecules and the cytoskeleton eg keratins. The epithelial integrity is reduced and permeability is increased after allergen challenge (5). The epithelial tight junctions were previously found to be altered in inflammatory bowel disease (6) and asthma (7). Expression of epithelial cell adhesion and cytoskeleton proteins were found to be reduced in out of season SAC compared to normal subjects and normal expression of e-cadherin is important for the maintenance of tight junctions and epithelial barrier function (8).

Protease activated receptor 2 (PAR-2) was found to be increased in SAC by means of immunohistochemistry (9). Increased PAR-2 expression is known to be related to increased epithelial permeability. In the same study the expression of adhesion molecules ICAM-1, VCAM-1 was assessed and was found that there was poor epithelial expression in both normal and “out of season” conjunctival tissues and ICAM-1 was found to have increased expression (though not statistically significant) in SAC. Therefore there are structural changes of the epithelium in allergic disease that alter the barrier properties and may relate to alterations in function as the epithelium becomes structurally weaker and more permeable.

1.3. Allergic inflammation of the airway and conjunctival mucosa

The role of the conjunctival epithelium is crucial in allergic inflammation and there are similarities with its role in atopy and the asthmatic lung. Asthma and rhinitis, may be associated with allergic conjunctivitis. The conjunctiva has been described as a representation of the upper extremity of the respiratory system which maintains a drainage system into the nose via the nasolacrimal duct (10). This can be demonstrated by the reduction of rhinitis symptoms by instillation of topical eye drops (11,12).

Allergic conjunctivitis can be reversed either spontaneously or with treatment, depending on disease severity. The diagnosis is a clinical one and in the absence of a standardized definition it is not possible to make clear evidence based management recommendations. The same applies to asthma where central to all definitions is presence of symptoms and of variable airway obstruction that can resolve spontaneously or due to treatment (13).

Seasonal and perennial allergic conjunctivitis involve a type 1 hypersensitivity reaction similar to the asthmatic subjects experiencing seasonal reversible disease. In the chronic forms of allergic conjunctivitis where corneal involvement is present, disease severity is greater and involves a Th2 cell mediated response, as seen in airway dysfunction in asthma (14). The most detailed investigation is that of the respiratory epithelium. Nasal and bronchial mucosa are able to express ICAM-1 and its levels correlate to disease severity (15-17) and similar properties were shown in the conjunctiva in ocular allergy as described above (18). Similarly HLA-DR is expressed by airway epithelial cells and is increased in asthma (15). Human nasal and bronchial epithelial cells express various cytokines with proinflammatory properties e.g. IL-1, IL-3, IL-6, IL-8, TNF α , RANTES, GM-CSF which are increased in allergic disorders (19,20) and decreased after anti-allergic treatment (21-23).

1.3.1. T cell derived cytokines and allergic asthma

It is well established that asthma can be considered mainly as a Th2 driven disease with IL-4, IL-5, and IL-13 participating in the immunopathogenesis of allergic inflammation associated with an Ig-E – dependent Type I hypersensitivity reaction and mast cells. Increased levels of Th2 cytokines, Ig-E levels, eosinophils and mast cells are reported in asthmatic lungs relative to controls (24,25). Atopic patients suffering from asthma had a Th2 type of airway inflammation with increase levels of IL-4+ and IL-5+ cells as well as increased eosinophils, and raised IgE levels in bronchial biopsies (26). On the other hand non-atopic asthmatics did not have evidence of Th2 inflammation but had raised levels of IL-8 and neutrophils without elevation of serum IgE. Increased mast cells numbers were observed in both non-atopic and atopic asthmatics regardless of IgE levels. Additionally the same group reported structural differences: there was greater epithelial damage in the atopic asthmatics (26). The diversity of symptoms in asthma has lead investigators to classify asthma into atopic and non-atopic as well as eosinophilic/non-neutrophilic/steroid sensitive and non-eosinophilic/ neutrophilic/ non-Th2-type asthma. Allergic or atopic asthma is considered as a Th2 driven disease with accumulation of eosinophils and mast cells.

There is good evidence that IL-17 may participate in the development of inflammation in asthma. IL-17 mRNA was found to be increased in the lungs and serum of asthmatics and the levels of IL-17 correlated to disease severity (27). IL-17 leads to epithelial and smooth muscle cells activation and potentiation of bronchial fibroblasts (28). IL-17 enhances ICAM-1, IL-8 (neutrophil chemoattractant) and G-CSF (important for neutrophil development) (29,30) levels secreted by epithelial cells. IL-17 mediated inflammatory mediators do not seem to contribute to Th2-mediated eosinophil driven allergic asthma development. There are further reports suggesting that IL-17 may contribute to the pathogenesis of non-atopic, non eosinophilic, neutrophil dominant, non-Th2 driven asthma (31,32). However there is evidence that IL-17 may participate in the Th2 cytokines mediated mouse asthma by acting in two phases: in the sensitization phase by promoting allergen specific T helper cells and in the induction phase and by inhibiting the local allergic response (33).

1.3.2. The roles of fibroblasts

Conjunctival fibroblasts are capable of promoting local inflammation by expressing eotaxin-1 in response to IL-4, IL-13 and TNF α (34). It has been shown that conjunctival fibroblasts cultured from VKC biopsies prolonged the survival of eosinophils in culture up to 5 weeks and suggested that they produce survival factors e.g. GM-CSF that reinforce eosinophilic persistence in the tissue (35,36).

TGF β along with Th2 cytokines (IL-4) stimulated the expression of VEGF protein and mRNA in human conjunctival fibroblasts (by means of PCR analysis) (37). Similarly IL-13 caused an increase in VEGF levels but was not statistically significant at the mRNA level. Other pro-inflammatory cytokines including IFN γ (Th1 cytokines) induced less VEGF production leading to the conclusion that VEGF production was more dependent on Th2 cytokine-mediated inflammation (37). Furthermore fibroblasts' biosynthetic capabilities are amplified by eotaxin and VEGF resulting in remodeling and perpetuation of the disease process. TGF β treatment of asthmatic fibroblasts *in vitro* resulted in increased expression of both eotaxin and VEGF (38). However, very little is known about the role(s) of VEGF in epithelial remodelling in ocular allergy.

Histamine treatment of conjunctival fibroblasts for 24 hours induced expression of proinflammatory cytokines, IL-1, IL-6, IL-8, and production of pro-collagen I (39). Additionally histamine reduced apoptosis of fibroblasts caused by serum deprivation. These data suggest that the role of fibroblasts extends from structural stromal functions and matrix production to effector cells of allergic inflammation (40).

The fibroblast being a major collagen production cell is also known to participate in remodeling. Matrix metalloproteinases (MMPs) are produced by connective tissue cells and macrophages and are inhibited by their natural inhibitors (Tissue Inhibitors Matrix Metalloproteinases- TIMPs). These enzymes participate in many pathological processes: wound healing, inflammation, cancer and angiogenesis (41). The balance between MMPs and TIMPs determines proteolysis, degradation of all components of the extracellular matrix and tissue invasion. It is known that MMP-1 degrades collagen I, III and V (major collagens found in conjunctiva and cornea) and MMP-9

degrades the basement membrane component collagen IV as well as participation in eosinophil migration (42).

In chronic allergic eye disease there is significant fibroblast activation and collagen deposition compared to acute reversible forms of ocular allergy with maximal cellular infiltration in the subepithelial area. Remodelling and scarring lead to the development of clinical signs, dry eye and corneal involvement. More specifically collagen deposition, mainly type I and III, occurs at the subepithelial space and forms a fibrovascular structure that sustains the giant papillae as seen in VKC. This remodelling effect suggests abnormal extracellular matrix homeostasis resulting in increased collagen production. Conjunctival remodelling involves degradation of extracellular matrix and simultaneous synthesis and deposition of new matrix (43). In the same study the authors reported increased expression of MMP-1 and MMP-9 in the tears of VKC patients compared to normal subjects. More specifically the higher levels of MMP-9 to MMP-1 also correlated with disease activity indicated by corneal involvement, giant papillae formation, signs and symptoms and the authors proposed that the origin of the MMP-9 may be the eosinophils. Eosinophil cell counts correlated with pro-MMP-9 tear levels and MMP-9 activity. Furthermore it was suggested that the corneal shield ulcers in VKC are mainly limited to the superficial stroma due to the TIMPs action and the re-epithelialization of ulcers could be due to raised levels of MMP (43).

1.4 Tissue remodelling

Tissue remodelling is an adaptive physiological phenomenon due to physical or hormonal stimuli featuring inflammation, cellular hypertrophy, proliferation, apoptosis, and extracellular matrix changes. The prolonged remodelling process causes pathological changes eg fibrosis in asthma, and ocular allergy. Tissue fibrosis is mediated by growth factors via transcription factor activations. Remodelling is a collective term that includes the changes in the amount and organization of the cellular and molecular components of the tissues, and is known to have deleterious irreversible effects contributing to disease severity. Its role in airway disease such as asthma and COPD has been described and the potential for therapeutic reversibility or prevention have also been suggested yet not completely understood. Similarly, in allergic conjunctivitis there are environmental insults as well as genetic susceptibility predisposing and leading to development of remodelling of the conjunctiva. However, the full impact of various remodelling mechanisms, on the conjunctival tissues is not completely understood.

The process of remodelling involves structural changes such as alterations in the composition, size, and organisation of the cellular and molecular components of the tissue. The attempted repair process follows multiple episodes of injury due to chronic inflammation. The structural changes of normal cytological architecture lead to alterations in function, clinical signs and symptoms. For example in airway remodelling, features include epithelial abnormalities, basement membrane thickening, extracellular matrix abnormalities, thickening of the smooth muscle cell layer, increased vascularity, hypersecretion and hypertrophy of the mucous glands and thickening of the airway wall (44).

More specifically in asthma, the epithelium is fragile, the basement membrane thickened, the smooth muscle cells enlarged and in chronic obstructive pulmonary disease (COPD) there is airway fibrosis and alveolar destruction leading to emphysema (45). Therefore there may be overlapping of remodelling features despite asthma and COPD being pathologically distinct diseases (46,47).

Patients with asthma and COPD and non-symptomatic smokers show epithelial injury and repair, with denuded epithelial areas and squamous metaplasia (45). Wound healing is a process that involves multiple cellular and molecular events characterised by the participation of platelets, neutrophils, macrophages and T lymphocytes that interact with the tissues and release growth factors, cytokines and fibrin to repair the wounded tissue. In the conjunctiva similar features have been reported in chronic conjunctival allergic and cicatrizing disease.

It is known that epidermal growth factor receptor (EGFR), as a marker of epithelial activation, is increased in the respiratory epithelium in severe asthma (48) and, in turn, leads to increased expression of pro-angiogenic and growth factors e.g. TGF- β , VEGF. TGF β activates the mesenchymal subepithelial cells to produce matrix and enhances proliferation and may promote epithelial-mesenchymal transition (49). In addition there is extracellular matrix deposition (ECM). Fibrosis and remodelling lead to irreversible structural changes and reduction in function. Fibroblasts are affected by inflammatory mediators produced by mast cells and eosinophils as seen in idiopathic pulmonary fibrosis, scleroderma and Crohn's disease. The following table 1.1 summarises the structural and functional changes in airway remodelling.

Structural changes in airway remodelling	Pathophysiological changes
Epithelial injury	Increased symptomatology
Basement membrane thickening	Disease perpetuation
Goblet cell hyperplasia	Decreased lung function
Matrix deposition	Airway hyperresponsiveness
Mucus metaplasia	Mucous hyperecretion
Subepithelial fibrosis	Oedema
Increased smooth muscle	Irreversibility of airway obstruction
Vascular dilation and angiogenesis	Death
Vascular remodelling	

Table 1.1: Features of airway remodelling (Modified by Figure 3 of reference 49)

1.4.1 Growth factors and soluble mediators in tissue remodelling

It has been reported, by means of *in vitro* experiments, that the concept of damaged bronchial epithelial cells production of PDGF, β FGF, and TGF β -1 reinforce fibroblast proliferation (50). Further to this conclusion it has been suggested that conjunctival epithelial cells and fibroblasts communicate through active growth factors and cytokines production leading to regulation of epithelial differentiation, wound healing and angiogenesis (51,52). The role of fibroblasts and collagen deposition has been investigated in inflamed conjunctival tissue and tears from patients with ocular allergy.

Less is known regarding the participation of potential remodelling candidates (e.g. EGFR and its ligands, VEGF) in various forms of ocular allergy, particularly in epithelial remodelling in CAED compared to reversible forms. VKC is the form most studied to date and mainly by means of immunohistochemistry. Investigators have described the distribution and expression of epithelial and stromal molecules in VKC conjunctival biopsies. The mechanisms of giant papillae formation are still not fully understood and are thought to participate in corneal involvement in the chronic forms of ocular allergy. Previous studies investigated the cellular and cytokine participation in conjunctival remodelling mainly in VKC but very few have concentrated on the study of epithelial remodelling markers. In addition no proposal has been made as to what promotes the remodelling processes in severe forms of ocular allergy. On the contrary the bronchial mucosa and epithelium have been studied extensively and it has been proposed that modified epithelial function promotes tissue remodelling and sustain persistent inflammatory responses as seen in chronic asthma.

1.4.2 Eosinophils and remodelling

It has been shown in the asthmatic bronchial mucosa, by means of immunohistochemistry, that there are increased numbers of VEGF positive eosinophils and macrophages contributing to increased airway thickness. Eosinophils produce important angiogenic mediators to include VEGF, TGF β , TNF α , IL-6 and IL-8 and it has been suggested that eosinophils induce angiogenesis *in vitro* and *ex vivo* providing evidence for a possible role of remodelling (53). Th2 cytokines (IL-13) and TGF β synergistically increase eotaxin expression in human airway fibroblasts suggesting that fibroblasts may participate in the exacerbation of allergic inflammation (54). Antigenic stimulation induces VEGF expression in bronchial mucosal epithelial cells (55). Eosinophils express TGF β and therefore may participate in tissue remodelling (56).

Eosinophils and IL-5 were shown to affect TGF β and remodelling. More specifically, in the remodelled airways of IL-5 deficient mice, TGF β was reduced (57). Similarly eosinophil TGF β expression contributes to airway remodelling in anti-IL-5 mAb - treated asthmatic subjects since it was observed that TGF β levels and TGF β positive eosinophils were reduced (58). Furthermore PDGF, FGF β and TGF- β 1 induce fibroblast proliferation and extracellular matrix production in pulmonary fibrosis (59).

1.4.3 The role of MMPs in remodelling

The distribution of MMP- 1, -3, -9, -13 and TIMP-1 in VKC, asthma and nasal polyps by means of immunohistochemistry was studied, aiming to show the potential contribution of MMPs to remodelling in different organs. In this comparative study, there were differences in collagen and MMP expression in the three different tissues. The differences in collagens' expressions reflects the obvious differences of the anatomical structures and the differences in MMPs expression may be a feature of different tissues undergoing similar processes in allergic inflammation (60). More specifically there was intense expression of collagen I in VKC giant papillae and nasal polyps which are fibrovascular outgrowths rather than just mucosal thickening as seen in asthma. Collagen III was increased in asthma and VKC suggesting that the remodelling processes may differ in tissues. It was also shown that there was

increased MMP-13 expression in the epithelium and moderate expression of MMP-3 and MMP-9 (60). Collagen I and III are those most involved in remodelling processes. Regarding MMPs, MMP-1, MMP-3, MMP-9 and MMP-13 were expressed in VKC, MMP-1 and -9 nasal polyps and MMP-13 was overexpressed in bronchial epithelium. MMPs are known to be increased in the bronchial mucosa in asthma especially during exacerbations (61) and increased tear levels and activity of MMP-1 and 9 were found to reflect clinical signs in VKC suggesting a strong role in allergic inflammation and remodelling (43,62). MMP-9 has been shown to be involved in eosinophil migration (63) and increased levels of MMP-9 and TIMP-1 were reported in bronchoalveolar lavage and sputum in asthma patients (64). MMPs interact with cytokines known to play a crucial role in allergic inflammation. More specifically MMP-9 is shown to catalyze the activation of IL-1 β (65) and TNF α (66) and hence have pro-inflammatory roles. Additionally MMP-9 can potentiate IL-8 (67) and process chemokines (68).

It has been suggested that in asthma there is also an altered expression pattern of extracellular matrix degrading enzymes. Asthmatic fibroblasts produce less MMP-3 (69) and MMP-2 (72). However MMP-9 was reported to be increased in asthmatic subjects which may account for the decreased expression of collagen IV in the basement membrane of asthmatic subjects (70).

The interactions of MMPs also induce mast cell activation. In fact MMP-1 was reported to be involved in mast cell infiltration and activation (71). The study reported that collagenase-1 was expressed by human mast cell cultures and by mast cells in normal and inflamed tissues. EGF receptor activation and VEGF are overexpressed together with a variety of other fibrogenic factors, resulting in production of collagens leading to epithelial-mediated remodelling (123). The levels of expression of different MMPs in VKC conjunctiva, nasal polyps and asthmatic airway have been investigated. These different tissues express at different levels of various MMPs whilst undergoing similar disease processes. There was increased MMP-13 expression in the epithelium and modulate expression of MMP-3 and MMP-9 (60).

1.5 Asthma and the Epithelial Mesenchymal Trophic Unit (EMTU) hypothesis

Atopy is a key characteristic of asthma pathogenesis and T-helper cell (Th2) airway inflammation participates in airway dysfunction. T cells, mast cells, and eosinophils are features of the Th2 response leading to an inflammatory response. The T-helper cell type-2 paradigm for asthma provides a link with atopy. Mast cells, eosinophils, and basophils coordinated by T cell antigen presenting cells have an important role in mediating inflammation in asthma. Atopy is an important risk factor for asthma but it accounts only for <40% of the attributable risk for having the disease (73). Corticosteroids administered via inhalation are shown to be efficacious by suppressing Th2 cytokines which mediate eosinophil and other immune system cells responses (74).

In chronic asthma there may be persistence of airway dysfunction despite high dose inhaled corticosteroids. This may be partially overcome with the addition of long acting inhaled β_2 adrenoceptor agonists providing relaxation of the smooth muscle cells and the corticosteroids suppress inflammation. Therefore in chronic asthma some disease components may not respond successfully to steroid treatment (75). This in turn leads to the conclusion that “there may be two mechanisms running in parallel that are essential to convert the disease from mild to severe and chronic:

- i) a susceptible airway linked to altered structure and function (remodelling), and
- ii) a microenvironment capable of sustaining a chronic inflammatory response due to an imbalance of EGF and TGF β signalling pathways e.g. mast cell population of airway smooth muscle cells correlating with variable airflow obstruction (76) and BHR and eosinophils in airway lumen correlating with asthma exacerbations (77)” (78).

It is suggested that an additional series of events is required to provide a better explanation for the disease chronicity and severity. This is based on the evidence that the altered epithelium communicates with the underlying mesenchyme to create a trophic unit that induces and reinforces remodelling from the epithelium. This is achieved with the participation of Th2 cytokines which maintain inflammation and drive remodelling (79).

1.5.1. Chronic asthma: altered response to injury and epithelial-mesenchymal communication

On its own the Th2 mediated inflammation is insufficient to cause asthma. The model of the chronic wound with production of growth factors involving activation of the epithelial-mesenchymal trophic unit (EMTU) has been proposed which provides the essential stimuli for altered airway wall structure and function (80). More specifically Holgate's suggestion states that the asthmatic state from a combination of increased susceptibility of the bronchial epithelium to injury and prolonged tissue repair involves an imbalance in responses to tyrosine kinase receptor ligands (EGF, TGF β) and Th2 cytokines. These in turn alter the cell-cell communication between the epithelium and the subepithelial mesenchymal cell layer leading to activation of the EMTU. The abnormal EMTU activation causes structural airway changes promoting chronic inflammation.

Communication between the epithelium and the underlying sub-epithelial fibroblast layer is reminiscent of the process that promotes branching morphogenesis in the fetus where the epithelium and mesenchyme function as a trophic unit (EMTU) (78). The EMTU plays an important role in pulmonary embryogenesis and its reactivation leads to pathologic remodelling in chronic asthma. It is established that during morphogenesis EGFR activation increases expression of MMP enzymes to degrade the extracellular matrix and induce epithelial proliferation. On the other hand TGF β expression reduces MMP levels and matrix degradation leading to inhibition of epithelial proliferation. The intermittent changes of EGF/TGF β balance produces alternating linear and branching of the developing airways (81).

Therefore this imbalance leads to the activation and function of tyrosine kinase receptor ligands involving epidermal growth factor (EGF) and transforming growth factor beta (TGF β). These molecules interfere and change the cell communication between the epithelium and the subepithelial mesenchymal cells. Epidermal growth factor receptor (EGFR) family members are upregulated as a normal response to epithelial injury aiming to promote proliferation and repair. In asthma EGFR expression is upregulated and correlates with disease severity irrespectively of the

inhaled corticosteroid treatment of the patient. Immunohistochemistry studies have shown that EGFR's expression is increased in the epithelium in asthma but not in the normal epithelium (82,83). This is illustrated in Figure 1.7 below:

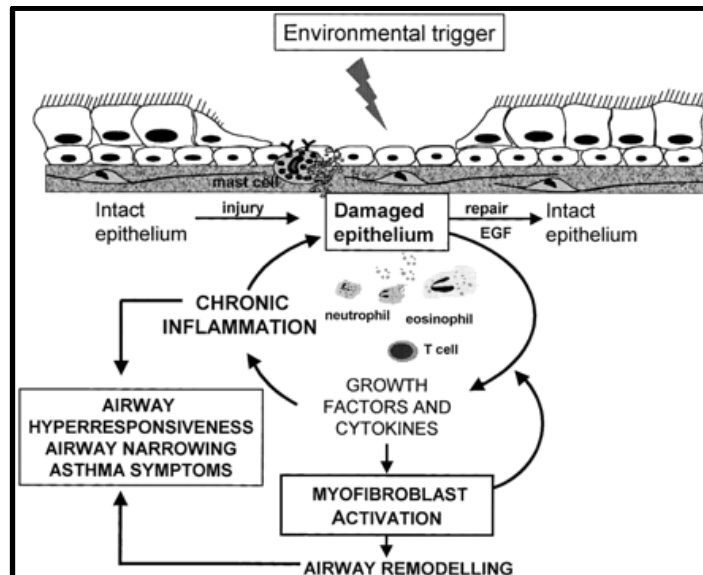


Figure 1.7: Activation of the epithelial-mesenchymal trophic unit in chronic asthma: injured epithelium and underlying fibroblasts induce mesenchymal cell proliferation leading to remodelling. Th2 cytokines (IL-4, IL-13) interact with the EMTU to enhance remodelling (78).

The authors propose this alternative paradigm to explain the rising trends and prevalence of the disease: changes in the environment may have exposed a pre-existing susceptibility involving local factors (78,84). In the case of chronic asthma it is proposed that in genetically susceptible individuals exposure to environmental factors closely associated to asthma (as opposed to atopy) causes a continuous activation of the EMTU leading to remodelling and chronic disease (78,84,85). These environmental factors may include exposure to environmental tobacco smoke, air pollutants, a diet low in antioxidants high in fat and proteins, pre- and perinatal exposure to oxidants (e.g. paracetamol) and respiratory virus infections (85). TGF β produced by the epithelium seems to play a role as well by differentiating airway mesenchymal cells into myofibroblasts which have contractile and secretory actions and provide a continuous source of precursor cells to generate smooth muscle cells (86,87). TGF- β 2 participates in airway wall thickening by inducing collagen secretion and promotes VEGF release (88,89).

1.5.2. Th1 and Th2 participation: essential but not enough to account for disease severity.

Based on the evidence described in the previous sections, cellular and molecular mechanisms of chronic and severe asthma are different from those of mild forms of the disease. The severe and chronic asthma requires early activation of epithelial-mesenchymal communication. On the contrary the mild forms of the disease are more dependent on a Th2 inflammatory response alone (78). Th2 cytokines IL-4 and IL-13 promote equally TGF β release in epithelial cells and in asthmatic fibroblasts they promote production of eotaxin, GM-CSF which might provide an explanation for the recruitment of eosinophils (38). Additionally IL-4 and IL-13 have been shown to increase TGF α production from the epithelium in asthmatic epithelial cell lines *in vitro* (90).

The trophic response leads to a chronic wound state and remodelling. In the remodeled airway mediators, cytokines and matrix agents promote an ideal microenvironment for perpetuation of chronic inflammation (91) adapting an additional Th1 pattern that involves participation of neutrophils and pleiotrophic cytokines e.g. TNF α , IFN γ which induce further tissue damage (92). It is also reported that Th1 cytokines participate in the development of childhood asthma (93,94). Th2 cytokines such as IL-4, IL-13, IL-9, GM-CSF have been localized and favored by the EMTU and maintain inflammation as well as driving remodelling processes (95,96). These interactions are illustrated in Figure 1.8 overleaf. More specifically, a parallel model for asthma pathogenesis in which inherited or acquired epithelial susceptibility to environmental agents leads to induction of stress-injury and repair. Growth arrest and prolonged repair enhances cell-cell communication in the EMTU leading to propagation of remodelling responses. As shown in figure 1.8 at each level the Th2 cytokines interact with the EMTU to amplify these responses (82).

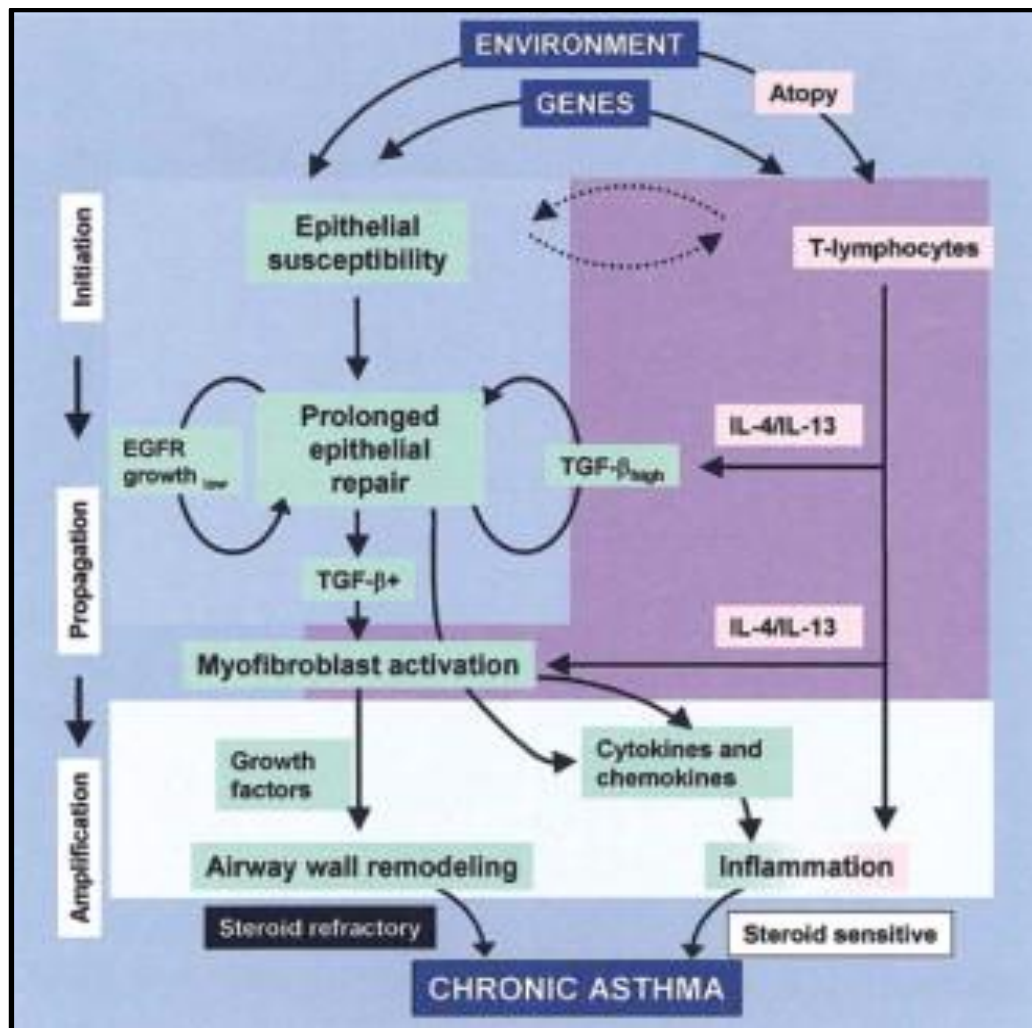


Figure 1.8: The interaction between Th2 mediated inflammation and EMTU in asthma pathogenesis (84)

1.5.3. Mechanisms of epithelial damage in asthma: epithelial-mesenchymal communication.

1.5.3.1 The role of growth factors and the EMTU hypothesis

EGFR is a glycoprotein (130kDa protein and 40kDa sugar chain) made of four domains: the glycosylated extracellular domain is the binding site for EGF and TGF α , a transmembrane lipophilic segment, tyrosine kinase domain and a phosphorylation domain. The binding of EGF or TGF α to the receptor causes DNA synthesis and cell proliferation/hypertrophy. The complete mechanism of the signal transduction is unclear and it is assumed that it involves tyrosine autophosphorylation and phosphorylation of interacting proteins. It becomes activated due to homo- or heterodimerization which is mediated either by ligand binding or by high receptor density due to overexpression. It is expressed by epithelial tissues and epithelium-derived tumours. It is reported that there is an asthma related increase in epithelial EGFR with remodelling suggesting a link between with subepithelial fibrosis and epithelial damage/activation (97). The EGFR expression levels are increased in asthma and correlate with disease severity (48). This overexpression is due to tissue response to injury but not proven to induce cell proliferation in asthma (98). The EGFR is known to be expressed in the basal surface of the epithelium of upper and lower airways as well as in bronchial epithelial derived cell lines (e.g. H292 and 16HBE14o) (99,100).

Members of the EGF family include TGF α , heparin binding EGF-like growth factor (HB-EGF), amphiregulin and epiregulin and are important regulators of epithelial population due to their ability to stimulate cell migration, proliferation, differentiation and survival (48). The EGF family of growth factors exerts its effects by binding and activating the EGFR. EGFR signaling was shown to be an intrinsic component of the response to injury and EGFR activation occurs rapidly after tissue damage and is independent of exogenous ligands (48). Epithelial EGFR expression occurs at areas of damage in asthmatic bronchial biopsies and more specifically there is disease-related increase in its expression. Corticosteroid treatment did not decrease the expression of EGFR in intact bronchial epithelium despite that corticosteroids are known to promote epithelial repair *in vivo*. This suggests a steroid non-

responsiveness element that may participate in severe chronic disease leading to the conclusion that increased EGFR expression blocks the epithelium into a repair mode which promotes inflammation and remodelling by provision of pro-inflammatory mediators, cytokines and fibrogenic growth factors (48). There is no similar evidence published so far for the severe forms of allergic conjunctivitis where steroids may also not be enough to achieve disease control.

EGF promotes mitosis of human bronchial epithelial cells and *in vitro* studies have shown that EGFR activation plays a significant role in airway epithelial repair after injury (98). However despite the increased EGFR expression in asthma, there is no increased epithelial cell proliferation to replace the lost columnar cells (98,101). Further studies attempted to provide an explanation and investigated the role of p21^{waf}, a cyclin dependent kinase inhibitor that was reported to be overexpressed in epithelial cells in patients with severe asthma (98).

TGF α shares a 33% homology with EGF and binds to the EGFR inducing activation of tyrosine phosphorylation and cell proliferation. TGF α is shown to be released at higher levels in asthmatic epithelial cells in response to pro-inflammatory cytokines e.g. TNF α , IL-4, IL-13 and allergen (90). It is expressed mainly by epithelial cells, macrophages and eosinophils. The bronchial epithelial cell line 16HBE14o expresses TGF α in addition to HB-EGF and it is proposed that these ligands are responsible for EGFR activation (48). In nasal biopsy specimens of normal volunteers exposed to ozone for two hours there was significant increase in the expression of EGFR, EGF, and TGF α with a positive correlation of EGFR epithelial expression and increase in neutrophil numbers. More specifically, EGFR expression was significantly increased *in vitro* 6 hours after exposure of the cells in TNF α and *in vivo* there was increased EGFR expression in nasal biopsies 8 hours after ozone exposure.

1.5.3.2 The role CD44 in enhancing EGFR signalling

CD44 is expressed by a variety of cells: T and B lymphocytes, monocytes, erythrocytes, epithelial cells, granulocytes, fibroblasts and mast cells. It is a glycoprotein transmembrane adhesion molecule (90kDa) which acts as a cell surface receptor for hyaluronate suggesting role in cell regulation and migration. CD44 is known to be upregulated in the respiratory epithelium in asthma and associated with epithelial injury (102,103) and its expression can be regulated by pro-inflammatory cytokines (104). CD44 has a physiological role in epithelial repair in airway epithelium derived cells (105). Cytokines including IFN γ increased cell adhesion via CD44 without increasing CD44 expression. Additionally CD44 mediated cell migration is involved in the response of airway epithelial cells to damage and promoted the epithelial cell repair process. High levels of CD44 expression were also observed on the cells close to the damaged area where epithelial migration takes place (104). It was suggested that CD44 may have an important function in localizing chemokine and growth factors to the disrupted epithelium (106).

There are four EGF receptor subtypes which are activated upon ligand crosslinking of two of these receptors (erbB B1 or B2) on cell surface. This activates tyrosine kinase which phosphorylates the opposite chain and leads to a cellular signal generation which has two directions:

- 1) into the nucleus via STAT-1/STAT-3 protein activation (signal transducer and activator of transcription) to produce cell proliferation
- 2) activation of alternative cell pathways involved in cell mobility linking to the actin cytoskeleton and turning an epithelial cell into a mobile mesenchymal cell (227).

In vitro investigations for the role of EGFR in damaged bronchial epithelial repair have shown that it becomes rapidly phosphorylated and the repair rate is accelerated by the addition of exogenous EGF (48). CD44 is suggested to play an interesting role in this mechanism which is illustrated below in Figure 1.9.

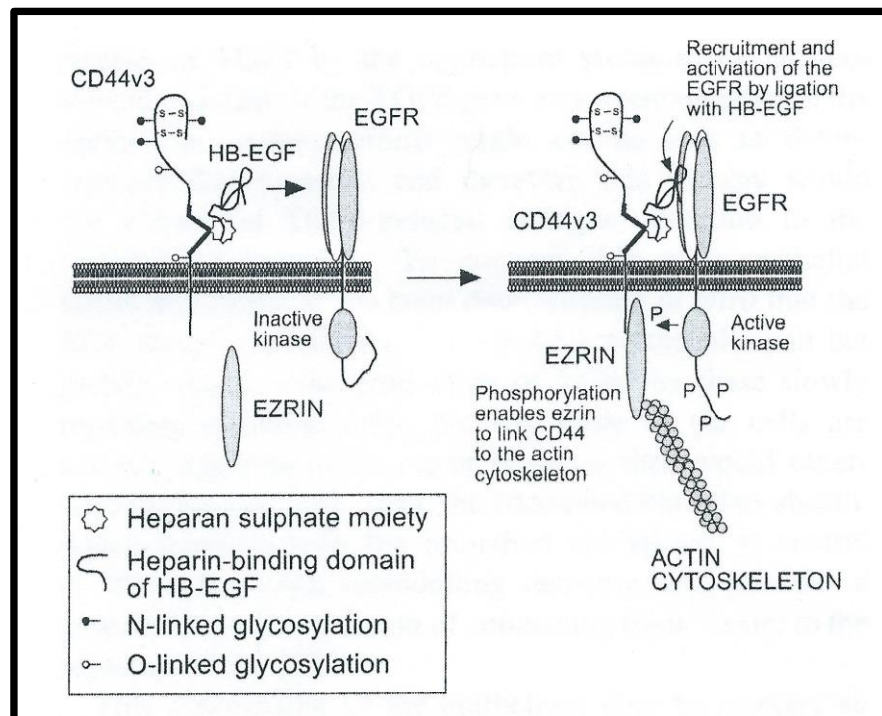


Figure 1.9: The role of CD44 enhancing EGFR mediated signalling and epithelial cell migration (106).

The epithelial 3v CD44 isoform selectively binds heparin binding EGF and when overexpressed in damaged or wounded epithelium it captures available EGF and presents it more efficiently to the cell surface EGFR. This in turn activates the kinase and promotes the epithelial repair. Additionally CD44 helps ezrin connect to moesin which is linked to the cytoskeleton and leads to cell movement as part of the repair process. The EGFR is overexpressed in injured epithelium and this has been reported to occur in the bronchial epithelium of patients with mild and severe asthma (100). The level of EGFR epithelial expression is disproportionate to the level of epithelial cell proliferation. Therefore in asthma increased EGFR expression does not lead necessarily to a repair response and a normal epithelium, as shown in vitro in primary bronchial epithelial cell cultures (16HBE 14-o-cells).

TGF β is also expressed at wound sites of the bronchial biopsy similar to EGFR but is independent to EGFR activation. This may suggest that there are parallel mechanisms taking place in the epithelium (48).

1.5.3.3 The roles of Transforming Growth Factor- β (TGF- β) and the TGF- β – EGF axis

There are three TGF β isoforms: β 1, β 2, β 3 and is secreted from inflammatory cells, epithelium, myofibroblasts, fibroblasts and can promote remodelling by inducing production of extracellular matrix components. TGF β participates in the homeostasis of lung immunity, lung development and extracellular matrix deposition. It mediates intracellular signalling via the Smad proteins (serine phosphorylated by the type I receptor after ligand activation) leading to transcription of target genes as shown in Figure 1.10 below. It is anti-inflammatory (107) and promotes cellular migration (108). On the contrary an excess of TGF β can inhibit epithelial proliferation that is essential for wound healing. In asthma it is shown that its expression is increased and which can account for the changes seen in airway remodelling.

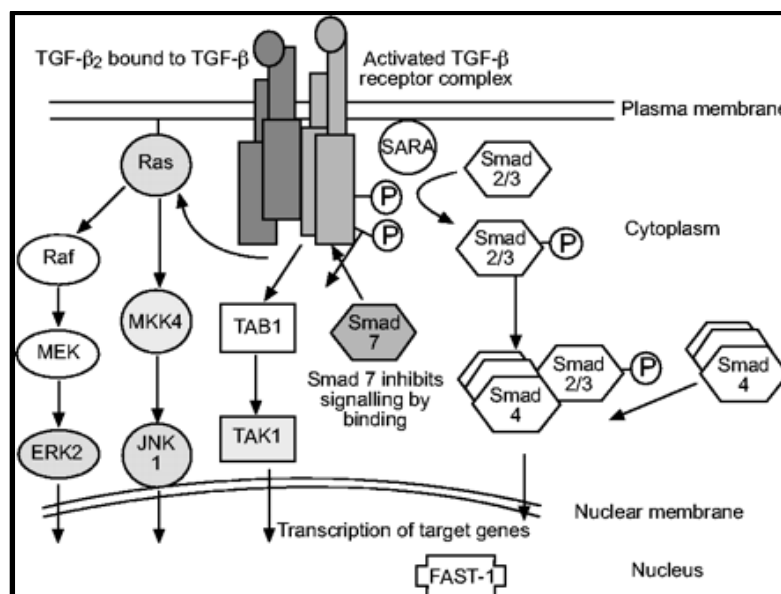


Figure 1.10: TGF β signalling via Smads resulting in transcription of genes e.g promotes fibronectin synthesis, collagen proteins (260).

TGF- β 1 regulates the migratory phase of epithelial repair (109). More specifically it can increase the migration of damaged bronchial epithelial cell monolayers (110). Additionally it has been shown to inhibit cell proliferation of epithelial cells (111) with upregulation of cyclin dependent kinase inhibitors (e.g. p21^{waf}) (112).

In the bronchial fibroblasts TGF β can exert a number of effects. TGF β promotes the transformation from fibroblast to myofibroblast (via Smad mediated adhesion independent and dependent signaling pathways) (113) and promotes their survival by inhibiting apoptosis mediated by IL-1 β (164). Myofibroblasts are transient *in vivo* and have more synthetic capabilities than fibroblasts, predominate in granulation tissue and play an important role in fibroproliferative disease when upregulated. It has been proposed that TGF β induces transformation of the myofibroblast to smooth muscle cells and in asthma the smooth muscle cell layer is hyperplastic (114).

Th2 cytokines (IL-4, IL-13) are shown to exert myofibroblast transformation indirectly via TGF- β 2 release (38). Additionally, TGF β was reported to upregulate fibroblast proliferation (115,116). Integrin mediated TGF β activation in EMTU was reported in foetal tracheal epithelial cells (117). It was suggested that this mechanism could result in delayed epithelial repair and stimulation of sub-epithelial matrix deposition and participation in airway remodelling.

TGF β and EGF promote various cellular events including growth, migration and lung embryogenesis. TGF β antagonizes EGF-induced attachment of cells on a fibronectin matrix (118). Additionally TGF β inhibits EGF- induced bronchial epithelial cell migration (119). Both molecules participate in lung morphogenesis: TGF β inhibits growth of the developing airways (120,121) whereas EGF promotes elongation of the bronchial tube (122). From the above we can conclude that TGF β can affect EGF signaling and vice versa and the outcome is determined by the relative balance between the two molecules. The balance between EGF-mediated proliferation and TGF β mediated fibroblast differentiation is of particular importance in the pathogenesis of airway remodelling.

1.5.3.4 Regulation of epithelial cell population: The role of p21^{waf} cyclin dependent kinase inhibitor

As mentioned earlier, the increased expression of EGFR in the asthmatic airway epithelium is not associated with increased epithelial cell proliferation. The bronchial epithelium acts as a physical barrier and serves in airways defense. Cell homeostasis is controlled by a balance between proliferation, inhibition of growth and apoptosis. Cell cycle inhibitors control cell proliferation. During the cell cycle entry into S phase is controlled by the cyclin dependent kinases (CDK). One example of CDK inhibitor is the antiapoptotic factor p21^{waf} which blocks G1 to S phase transition by inhibiting the phosphorylation of retinoblastoma protein (124). p21^{waf} can be induced by cytokines with antiproliferative properties TGFβ, IFNγ, by corticosteroids and by injury (125).

p21^{waf} expression has been mainly localized at the epithelium and has been found to be greater in asthmatic subjects and unaffected by corticosteroid treatment. p21^{waf} was induced *in vitro* by TGFβ or H₂O₂ but not dexamethasone. The increased expression of p21^{waf} in asthmatic bronchial epithelium may reflect its inability to proliferate leading to an imbalance between proliferation and apoptosis and may contribute to persistent inflammation and remodelling in asthma (98). Furthermore fibroblasts' biosynthetic capabilities are amplified by eotaxin and VEGF resulting in remodelling and perpetuation of the disease process. TGFβ treatment of asthmatic fibroblasts *in vitro* resulted in increased expression of both eotaxin and VEGF.

1.5.3.5. EGFR and growth factors in chronic allergic eye disease

The normal epithelium in the conjunctiva may show weak immunoreactivity of VEGF and EGFR but not for TGF β , PDGF, β FGF (123). The conjunctival epithelium overexpresses EGFR and VEGF as shown in Figures 1.11 and 1.12 (126). The increased immunoreactivity of EGFR is shown to be more intense in the deeper epithelial levels and VEGF in all levels (biopsies from 16 VKC patients with severe active disease in King Abdul-Aziz University Hospital, Saudi Arabia) but some variations were also noted. More specifically EGFR positive inflammatory cells were observed in 8 out of 16 biopsies and VEGF positive inflammatory cells were observed in 9 out of 16 biopsies. Additionally inflammatory cells (eosinophils, macrophages, monocytes) expressing TGF β , EGFR, VEGF, β -FGF, PDGF were located in the stroma (126).

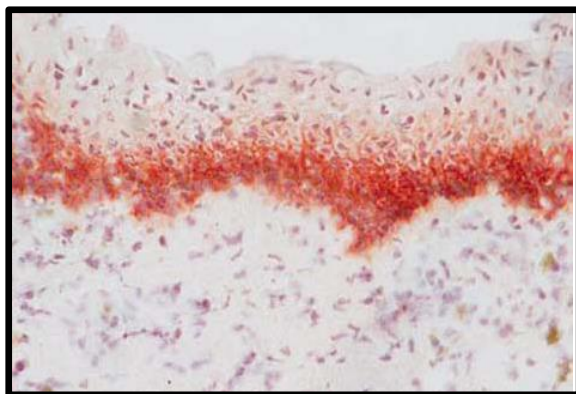


Figure 1.11: EGFR expression in VKC is stronger in the deeper Epithelial layers (126)

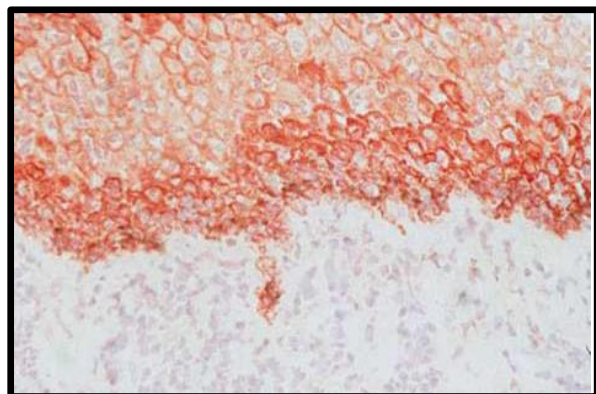


Figure 1.12: The epithelial expression of VEGF in VKC (126)

Expression of integrins $\alpha 3$ and $\alpha 6$ was found increased in the basal and sub-basal epithelial areas in VKC. Growth factors can modulate the level of expression of integrins. Integrins have been documented to participate in the maintenance of epithelial cell populations, in epithelial wound healing and remodelling (127-129). These results suggest a possible role of growth factors in remodelling in the pathogenesis of VKC by means of immunohistochemistry studies.

Fibroblast proliferation or fibrosis occurs in chronic inflammatory disease and in chronic ocular allergic diseases. The giant papillae in VKC form the pathological construction of fibroblast activation and tissue remodelling. Collagens I, III, VII and fibronectin were highly expressed in the sub-epithelial area in VKC with collagen I being the major element in VKC compared to controls (130). There is increased diffuse epithelial expression of fibrogenic growth factors TGF β , PDGF, and FGF in association with increased deposition of collagen in the giant papillae of VKC (130). Collagen VII expression is considered an indication of increased epithelial growth and conjunctival area and collagen I seemed to be the major connective tissue component in VKC. The epithelium in VKC subjects was intensely positive for PDGF, FGF β , and TGF- β 1 suggesting that they are produced by the epithelium or they have been accumulated there from tears or other sources. Additionally it was suggested that these factors may be involved in the chemoattraction of mast cells.

TGF β is an important regulator of extracellular matrix deposition by controlling collagen and protease inhibitors expression (131,132). It is involved in the regulation of allergic inflammation and remodelling. Intracellular signalling by TGF β members is mediated via binding to a heteromeric receptor complex (130,131,133) causing phosphorylation of its intracellular receptors (Sma- and Mad- related protein (Smad) family of signal transducers (131,132,134). TGF- β 1 and - β 2 levels were significantly increased in association with Smads (131).

There is no published data to date regarding the roles of CD44 in ocular allergy, as an enhancer of EGFR signalling, and TGF α as a major ligand binding the EGFR. Similarly there is no published data on the roles of p21, an anti-apoptotic molecule, in ocular allergy. Its overexpression in the epithelium may indicate an imbalance between proliferation and apoptosis and may contribute to persistent inflammation and remodelling in chronic allergic eye disease, as reported in chronic asthma. Although there is some data on the detection of growth factors participating in remodelling in VKC most studies concentrated on the roles of fibroblasts and there is no suggestion as to why it occurs in chronic allergic eye disease which could account for disease severity.

1.6 The role of the conjunctival epithelium in ocular allergy

1.6.1 Cellular participation and cytokines

Various cells participate in the immune response involving cytokine production, further chemotaxis and hypersensitivity. In VKC there is a massive involvement of T cells, macrophages, eosinophils and neutrophils which differentiates it from SAC or PAC as shown in Table 1.2 below that illustrates the various cells participating in the pathogenesis of each form of ocular allergy (135).

	IgE	Mast cells	Eosinophils	Th1 response	Th2 response	Corneal involvement
SAC	+++	+++	+	—	+	—
PAC	+++	++	+	—	+	—
VKC	+	++	+++	+	+++	+++
AKC	+	++	+++	++	+++	+++
GPC	—	+	—	+	+	—
CDC	—	—	+	+++	—	—

AKC, atopic keratoconjunctivitis; CDC, contact or drug-induced dermatconjunctivitis; GPC, giant papillary conjunctivitis; IgE, immunoglobulin E; PAC, perennial allergic conjunctivitis; SAC, seasonal allergic conjunctivitis; Th, T helper; VKC, vernal keratoconjunctivitis

Table 1.2: Overview of the cells and mechanisms in ocular allergy.

Mast cells contribute to the immunopathogenesis of allergic eye disease and are able to store produce and secrete several factors such as histamine, $\text{TNF}\alpha$, IL-4, IL-6, IL-8 contributing to the inflammatory response by the recruitment of eosinophils and neutrophils. Two forms of mast cells are present: the tryptase and chymase (MC-TC) and tryptase only (MC-T) found in mucosal membranes which increases in ocular allergy and is present in all epithelial layers. The conjunctival mast cell is central in the allergic cascades in ocular allergy. In the late phase of the allergic response after mast cell activation, the conjunctiva is infiltrated by inflammatory cells (neutrophils, lymphocytes, basophils, eosinophils). They are the principal inflammatory cells in SAC with eosinophilic recruitment capabilities. So far it is recognized that in addition to T-cells, mast cells, eosinophils and epithelial cells are significant sources of cytokines in allergic inflammation. There are different cytokine profiles, T cells and eosinophils in the epithelium of the conjunctiva in different forms of ocular allergy (18).

1.6.2 The pro-inflammatory roles and antigen presentation capabilities of the conjunctival epithelium

The conjunctival epithelium is a non-keratinized, 2-6 thick cell layer and plays an important role in the immunopathogenesis of ocular allergic disease. It contains goblets cells secreting mucin and it has the ability to express co-stimulatory molecules. However, so far the most detailed investigation of epithelial cells is that of the airway epithelium. The most important role of the epithelium of the conjunctiva, in ocular surface defense and inflammation, has long been thought to be its barrier function and stabilizing the tear film (136,137). The airway mucosa and the conjunctival epithelium, express adhesion molecules, surface antigens and pro-inflammatory cytokine mediators (138). So far there are reports on the cytokine profiles in allergic eye disease in relation to the role of the epithelium assessed by means of immunohistochemistry, flow cytometry and cell line cultures. It is known that the conjunctival epithelium plays a crucial pro-inflammatory role in the immunopathogenesis of chronic allergic ocular disease (139).

In SAC the predominant mediator is histamine produced via a type I hypersensitivity reaction with symptomatology reflecting allergen exposure. Immunohistological evidence in SAC conjunctival bulbar and tarsal biopsies showed increased expression of ICAM-1 and E-selectin compared to controls only during the pollen season (140). In VKC and AKC the mechanisms are more complex and in addition to the eosinophils, mast and epithelial cells, involve Th2 T cells.

Further immunohistochemistry studies on conjunctival biopsies from AKC, VKC, GPC patients revealed that the epithelial cells are capable of ICAM-1 adhesion molecule expression for leucocyte migration, MHC class II (HLA-DR in human) expression important in antigen presentation and T cell activation and synthesis of prostaglandins and leukotrienes essential for leukocyte activation and attraction (65). ICAM-1 interacts with LFA-1 to promote the migration, recruitment and retention of leukocytes. HLA-DR expression is also increased in chronic inflammatory conditions of the conjunctival epithelium which is required for antigen presentation to T cells with class II MHC (139). Its expression was increased in AKC and VKC more than GPC where there is no corneal involvement. Interestingly GPC is not considered a

true allergic conjunctival disease. It is caused by repeated mechanical irritation (e.g. contact lens use) and can be reversible upon removal of the cause. T cell infiltration is also seen in this form of chronic allergic eye disease. It is not associated with atopy.

The apical side of the epithelial layer is exposed to the environment and allergen directly whereas the basolateral surface appears to be the site of CD4⁺ T cell recruitment via a not well understood mechanism (141). T cell infiltration appears to be in all forms of chronic allergic eye disease: GPC, AKC, VKC (142). It has been reported that there are Th2 cytokine producing cells in the sub-epithelial layer in GPC, AKC and VKC. More specifically in AKC conjunctival T cell lines produce increased IFN γ , IL-10, IL-13 and little IL-4 and IL-5. (143) In VKC conjunctival cell lines produce IL-5 and IL-13 (141). Table 1.3 below summarizes the various cytokines known to participate in various forms of ocular allergic disease in relation to the various types of inflammatory cells involved (135). However, there has been no study to date to suggest the potential involvement of growth factors /or relate their detection to the severe forms of disease as opposed to the reversible IgE mediated types (SAC, PAC).

	IgE mediated (SAC and PAC)	IgE and non-IgE mediated (VKC and AKC)	Non-IgE mediated (GPC and CDC)
Mediators	Histamine Prostaglandin D2 Leukotriens PAF ICAM	Histamine Prostaglandins Leukotriens PAF ICAM, VCAM MMPs, proteases, MBP, ECP	Prostaglandins Leukotriens ICAM
Cytokines and chemokines	IL-1, IL-4, IL-8, TNF α , IFN γ MIP-1 α	IL-1, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, TNF α , INF γ Eotaxin, MCP, RANTES, TARC, Mig	IL-1, IL-6, IL-8, TNF α , INF γ RANTES, MCP
Cells	Mast cells Neutrophils Eosinophil	Mast cells Neutrophils Eosinophils Basophils T cells Macrophages NKT	T cells Macrophages Neutrophils

AKC, atopic keratoconjunctivitis; CDC, contact or drug-induced dermatoconjunctivitis; ECP, eosinophil cationic protein; GPC, giant papillary conjunctivitis; ICAM, intercellular adhesion molecule; IFN γ , interferon γ ; IgE, immunoglobulin E; IL, interleukin; MBP, major basic protein; MCP, monocyte chemoattractant protein; MIP-1 α , macrophage inflammatory protein 1 α ; MMP, matrix metalloproteinase; NKT, natural killer T cell; PAC, perennial allergic conjunctivitis; PAF, platelet-activating factor; SAC, seasonal allergic conjunctivitis; TARC, thymus and activation-regulated chemokine; TNF α , tumor necrosis factor α ; VCAM, vascular adhesion molecule; VKC, vernal keratoconjunctivitis.

Table 1.3: Tear mediators, cytokines and cells detected in *active* ocular allergies (135).

It has been proposed that due to their expression of co-stimulatory molecules and secretion of pro-inflammatory cytokines, these cells could present and process antigen. Additionally it was found that in chronic allergic eye disease there is an upregulation of various cytokines in the conjunctival epithelium: RANTES, GM-CSF, IL-3, IL-8, TNF α . There are different cytokine profiles in epithelial cells in different clinical disorders.

VKC is a Th2 driven disease with a Th2 cytokine profile: increased levels of IL-3, IL-4, IL-5 and IL-13. Th2 lymphocytes induce IgE hyperproduction (IL-4, IL-9, IL-13), recruitment of mast cells (IL-3) and eosinophils (IL-5) (141). The IgE mediated hypersensitivity reaction alone is not enough to explain the complete spectrum of progression and severity (144-146). So far it is known that the conjunctival infiltration of inflammatory cells leads to the release of toxic mediators (eosinophil neurotoxin, eosinophil peroxidase, matrix metalloproteinases 2, 9 which in turn causes corneal epithelial damage (147-151). In the epithelial cells TNF α , IL-1 β induce increased expression of ICAM-1 and IL-1 β is a more potent stimulator releasing IL-8 than TNF α . RANTES production requires IFN γ and TNF α treatment and is one of the most potent eosinophilic chemoattractants with effects to T cells and macrophages (152).

Dendritic cells, are potent initiators of primary immune responses, stimulate naïve T cells and participate in maintaining and initiating the Th2 response. They express the high affinity IgE receptor (Fc ϵ RI) that enables antigen presentation to T cells. In conjunctival biopsies from non-atopic and VKC patients it was reported that the Fc ϵ RI on dendritic cells is increased, which could enable antigen presentation and development of ocular symptoms. These cells had greater populations located in the epithelium (153).

1.6.3 The roles of eosinophils in ocular allergy

Levels of eosinophilic activation, and not total cell number are greater in those forms of ocular allergy with corneal involvement than without. There are also differences in the cytokine profile which may be attributed to the T cell subsets. It seems that the eosinophils have an important role and these may be either secondary to the actions of the various T cell subtypes or a cause affecting T cell responses (154). The enhanced activation of eosinophils in AKC and VKC suggested that these cells release their cytokines and could play a role in disease perpetuation (154). In AKC, there is increased eosinophil expression of IL-4, IL-8 and GM-CSF. IL-4 is a B cell stimulator and influences the development of T cells, whereas IL-8 is mainly a neutrophil chemoattractant (154). Other studies confirm that in VKC there is expression of IL-3, IL-5, IL-6, GM-CSF which are pro-inflammatory and chemoattractant for cells such as mast cells and B cells (155-157).

These immunohistochemistry studies enable the eosinophil cytokines to be studied because like mast cells eosinophils contain a significant amount of stored cytokines which are secreted upon activation. On the contrary, T cell cytokines studies require *in situ* hybridization since T cell cytokines are released almost immediately from the cells following activation.

1.6.4 The roles of T cell subsets

Mast cells numbers are increased in chronic allergic eye disease but the major cellular participants are mainly CD4⁺, CD8⁺ T cells and eosinophils and are found in increased numbers in the subepithelial areas of the conjunctiva. Epithelial cell-derived cytokines are detected in the conjunctiva in chronic ocular allergic diseases with different cytokine profiles in each form of disease (158). Studies have shown that in VKC there are increased number of activated T cells mainly located in the sub-epithelial layer and increased HLA-DR expression (159). T lymphocytes play an important role in mediating the immunopathology seen in the chronic allergic response in the skin and the lungs. The prevalence of T cells and their subsets in different chronic allergic eye conditions by means of immunohistochemistry has been investigated and it has been reported that there are significantly increased numbers of CD4⁺, CD45RO⁺, and HLA-DR⁺ T cells in the conjunctiva of patients with atopic and vernal keratoconjunctivitis and giant papillary conjunctivitis, compared to non-inflamed controls, with a corresponding upregulation of markers present on antigen presenting cells (158).

The cytokine profile of T cells in different clinical groups of subjects with chronic allergic eye disease (AKC, VKC, GPC) and normal control subjects was investigated by means of in situ hybridization to identify cytokine messenger RNA (mRNA), and two-colour immunohistochemical analysis. Allergic tissue expressed increased levels of mRNA for IL-3, IL-4, and IL-5 when compared with normal tissue (160). There was significantly greater IL-2 mRNA expression in subjects with AKC than in those with VKC and GPC. Immunoreactivity for T-cell IL-5 was present more frequently in subjects with VKC, AKC and GPC compared to normal conjunctival biopsies (159). However, T-cell associated IFN γ protein expression was greater in subjects with AKC than in subjects with VKC and normal individuals suggesting a Th2 T-cell cytokine array in subjects with VKC and GPC but a shift in cytokine profile toward a Th1 pattern, potentially because of differences in chronicity of the disorders, in subjects with AKC. These important functional T-cell variations in chronic allergic eye conditions are likely to be important in understanding differences in clinical characteristics and therapeutic responses (159).

T cells infiltrate the conjunctiva in AKC and VKC. The role of the Th1/Th2 paradigm was described in previous section. Further to this concept, there is growing evidence that other T cell subsets exist which might participate in allergic inflammation but little is known about their potential roles in ocular allergic disease. The identification of novel T helper cell subsets i.e. Th17, Th9, Th22 and regulatory T cells (Treg) resulted into a more detailed insight of the immunopathogenesis of autoimmune diseases and immune responses. Furthermore, these developments have led to a new approach and revision of the previously described Th1/Th2 paradigm, which provides the basic understanding the molecular immunopathogenic mechanisms of disease development. Figure 1.13 shows an up to date summary of IL-17 activities (164)

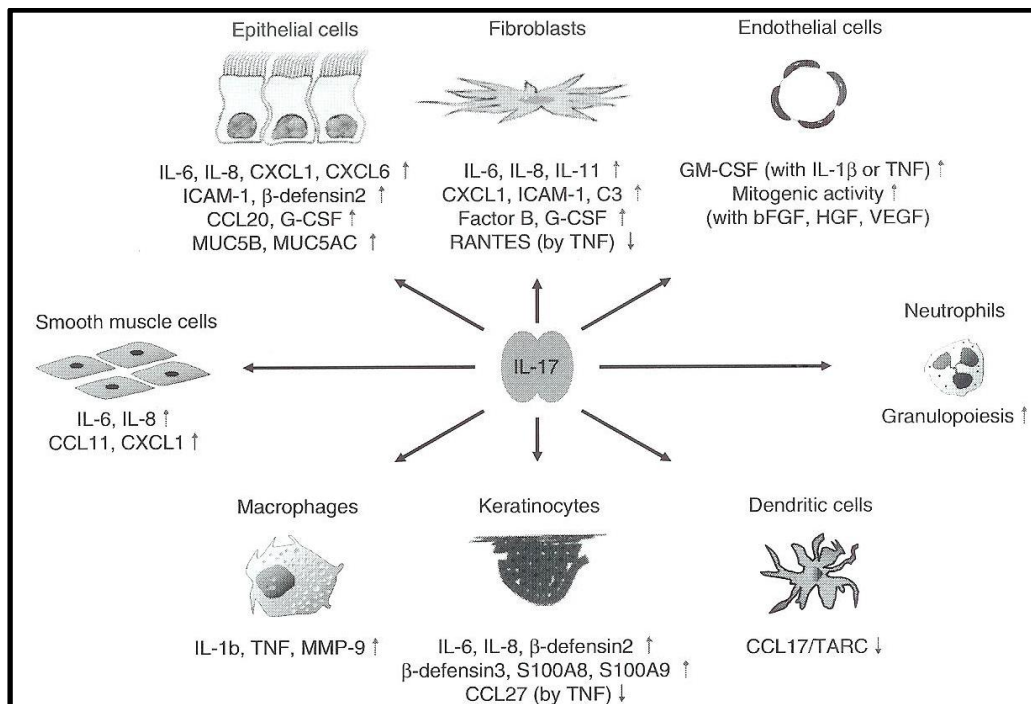


Figure 1.13: The effects of IL-17 on various types of cells (164)

IL-17 mRNA was found to be increased in the lungs and serum of asthmatics and the levels of IL-17 correlated to disease severity (162). IL-17 leads to epithelial and smooth muscle cell activation and potentiates fibroblasts. IL-17 enhances ICAM-1, IL-8 levels, G-CSF, CXCL-1 levels by epithelial cells. IL-17 mediated inflammatory mediators do not seem to contribute to Th2-mediated eosinophil driven allergic

asthma development. Allergic or atopic asthma is considered as a Th2 driven disease with accumulation of eosinophils and mast cells. There are further reports that support the fact that IL-17 may contribute to the pathogenesis of non-atopic, non eosinophilic, neutrophil dominant, non-Th2 driven asthma. To conclude, the main effector cytokine produced by Th17 cells is IL-17 which has six members (A-F) and is known to induce the production of IL-1 β and TNF α which have pro-inflammatory roles in the immunopathogenesis of allergic inflammation (162).

There is no published evidence for an important role of IL-17 in ocular allergic disease (163). On the contrary it seems that IL-17 plays an important role in experimental induced dry eye in mice (EDE) (163). In a recent study using an acute form of experimental allergic conjunctivitis - EAC (induced by ragweed pollen in Balb/c mice), severity of the disease did not differ in the naïve wild type or the IL-17 knockout mice suggesting that IL-17 is not important in acute allergic conjunctivitis. In the latter model, EAC developed due to increased expression of Th1 and Th2 cytokines (164).

Unfortunately there are no models of chronic allergic conjunctivitis currently available to address the role of different cytokines including IL-17, in chronic forms of conjunctivitis. Nevertheless it has been found in other experimental models eg experimental autoimmune uveitis, that blocking one or even two cytokines results in disease being mediated by other pro-inflammatory cytokines (165) suggesting these are compensatory mechanisms and disease can be mediated by several pro-inflammatory cytokines. In Experimental Dry Eye (EDE) when IL-17 was neutralized with anti-IL-17 antibody, the clinical signs were significantly reduced compared to controls (166). There is significant evidence that IL-17 contributes to ocular surface inflammation and tissue damage in EDE (166,167). The full spectrum of its roles in chronic allergic eye disease is yet to be determined. Similarly there is no data to date to suggest potential roles of IL-17 in tissue remodelling in ocular allergy.

1.7 The immunopathogenesis of ocular allergy

1.7.1 T cell roles

Historically it is known that the ratio of Th1/Th2 type cytokines plays an important role in the allergic inflammation development. The Th2 cell type, CD4⁺ T cells and Th2 cell cytokines predominate in VKC and these findings have been described since 1990s (168-171). On the contrary Th1 type lymphocytes protect against allergic disease by dampening the activity of Th2 by inhibiting the development and proliferation of Th2 cells. Additionally, IFN γ a typical Th1 cytokine, inhibits IgE synthesis (172-174).

Th2 T-cell lymphocytes are suggested to play a crucial role in the pathogenesis of allergic disorders by producing regulatory and pro-inflammatory cytokines (IL-4, IL-5, IL-13). Various studies provide evidence that increased levels of activated eosinophils are found in the tears and conjunctiva of patients with VKC and AKC and release pro-inflammatory and epitheliotoxic mediators after recruitment and activation by Th2 cytokines and chemokines (151,175-179).

On the other side the Th1 profile cytokines seem to be participating in AKC (168,170,171,180-182) but it is not clear if IFN γ plays a downregulating role or participates in the pathogenesis of allergic disease of the conjunctiva (183). IFN γ measured in the tears of patients with AKC and VKC was found to be increased in those with corneal damage and significantly correlated with the corneal score (183). This can suggest that increased expression of IFN γ , a pro-inflammatory cytokine, might be related to the development of disease severity of allergic inflammation. IFN γ upregulated ICAM-1 expression and production of IL-6, IL-8 (pro-inflammatory cytokines) suggesting that IFN γ may play an important role in inflammatory cell recruitment, attachment and activation. IFN γ may participate in the active phase of the disease of severe ocular allergy and IFN γ secreting cells may participate in the perpetuation of chronic inflammation since this cytokine up-regulated the expression of adhesion molecules, chemokines and co-stimulatory factors produced by conjunctival and corneal epithelial cells.

Using the mouse model (Brown Norway rats) it was shown that IFN γ downregulated the development of allergic conjunctivitis during the induction phase in the experimental model (184). IFN γ may have a downregulatory role in the induction phase of experimental conjunctivitis but in another study of experimental conjunctivitis in IFN γ knockout mice it promotes its development during the active phase (185,186).

Another factor affecting the Th1/Th2 balance is the expression of intracellular regulatory signals. The suppressor of cytokines SOCS3 and SOCS5 are expressed in Th2 and Th1 cells respectively and reciprocally inhibit the Th1 and Th2 cell differentiation. SOCS3 is expressed in the allergic conjunctiva and its inhibition reduces the severity of experimental allergic conjunctivitis (183).

1.7.2 Pathogenesis of IgE – mediated diseases: seasonal and perennial allergic conjunctivitis.

It occurs in sensitized individuals and the major pathogenic mechanism involves a type 1 hypersensitivity reaction initiated by allergen causing cross-linkage of the membrane bound IgE leading to mast cell degranulation causing the production of various cytokines and inflammatory mediators. There are two phases of the Ig-E Type 1 reaction: an early immediate phase which occurs minutes after activation and is caused by the degranulation of the mast cells releasing mediators as described above and a late phase occurring 4-6 hours due to chemoattraction of T cells and eosinophils. Histamine is one of the major molecules participating in the early development of symptoms. The activation of mast cells leads to the release of inflammatory mediators as well as *de novo* synthesis of cytokines, and eicosanoids. This response lasts 20-30 minutes and induces increased tear levels of histamine, tryptase, prostaglandins, leukotrienes (151). Phospholipase A2 (PLA2) is important in allergic conjunctivitis since it hydrolyses the acyl-ester bond and generates arachidonic acid which is then converted to prostaglandins and leukotrienes (187).

In SAC the symptoms occur in spring and autumn whereas in PAC they can occur all year round. SAC is more common and associated with pollen. PAC is related to animal dander, house dust mite or other allergens present throughout the year. Various pro-inflammatory cytokines are present with variable immune-mediated effects e.g. TNF α , IL-1 β , IFN γ after conjunctival epithelium activation and lead to increased production of ICAM-1 and RANTES.

The late phase of conjunctival allergic response occurs a few hours after the first mast cell activation. There is infiltration of inflammatory cells eg neutrophils, lymphocytes, eosinophils, basophils and an increased expression of adhesion molecules eg ICAM-1 and E-selectin. The adhesion molecules are found to be downregulated in the conjunctiva of patients with SAC out of season suggesting that the epithelium may be capable of self-protection that affects allergic disease progression (188).

Conjunctival mast cells are tryptase and chymase positive and produce IL-4 which promotes T-cell growth, Ig-E production from B-cells, upregulating adhesion molecules and regulating Th2 cell differentiation (189). IL-4 also induces eotaxin expression from conjunctival fibroblasts and keratocytes but not from the epithelium (190). The mast cells and their products participate in the pathogenesis of SAC and during the disease their numbers are increased (191).

In PAC there is also a Type I Hypersensitivity reaction but since the allergens are present continuously there is a more chronic inflammation. Mast cells are also increased in the conjunctiva in patients with PAC (192). Additionally neutrophils, eosinophils and T cells are also detected suggesting that other cell mediated mechanisms, though not clear to date, are involved. The changes in PAC are more pronounced than in SAC since there is prolonged exposure to allergens. In SAC and PAC there is no corneal involvement and the inflammation may resolve once the allergens are removed.

1.7.3 Current understanding of the immunopathogenesis of Atopic and Vernal keratoconjunctivitis.

More complex mechanisms are responsible for the development of these chronic forms of the disease and there is a persistent T cell infiltrate. In both diseases the T cell is considered the primary effector cell. The pathogenesis involves production of various cytokines by effector cells whose immunopathogenic roles are still under investigation.

Various cytokine profiles and immune cell types lead to inflammatory cell participation by production of mediators which in turn promote disease progression. The initiating mechanism in the chronic forms of ocular allergic diseases (AKC/VKC) is not yet fully understood. The major effector cells in AKC are the mast cells, T cells, eosinophils and conjunctival epithelial cells (193,194) and there is a Th1 cell response (168). A Th2 response is also contributing but to a lesser degree. The Th1 component involves a delayed hypersensitivity reaction and cell mediated cytotoxicity via a variety of cytokines including IL-2, IFN γ and IL-12. Additionally IL-8 and RANTES are also expressed in the epithelium leading to T cell, eosinophil and neutrophil chemoattraction (194).

However different patterns of T cell cytokines are observed in each disease. In VKC there is increased IL-5 and a Th2 cell profile. Eosinophils, fibroblasts and Th2 T lymphocytes contribute to this immune mediated allergic response. Th2 T-cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 and regulate IgE production, and induce significant antibody responses and eosinophil activation or differentiation (168). The role of the conjunctival epithelium is thought to play an important role in the immunopathogenesis of ocular allergic disease.

It has been shown that there are functional variations and differences in the cytokine production profile in both T cells and eosinophils in the different forms of chronic allergic eye disease, which may determine the development of corneal involvement.

GPC is histologically similar to AKC and VKC but there is no corneal involvement and no known etiology. It is not characterized as a “true” allergic disease as the other forms of ocular allergy and is thought to be triggered by persistent mechanical irritation (e.g. from the contact lens, ocular prosthetic implant, sutures in contact with the conjunctiva) and augmented by associated allergy. It can occur with any type of contact lens and the lens coating and trauma to the conjunctiva are probably the triggering factors. T cells do not seem to participate in its pathogenesis since T cell lines from GPC produced only small amounts of cytokines. Eosinophils are known to be involved but eotaxin is not participating significantly in its pathogenesis.

1.7.4 Giant papillary conjunctivitis: Non-IgE mediated “allergic” disease

GPC is suggested to be an inflammatory disorder that typically leads to giant papillae development on the superior tarsal conjunctiva. There is an immune response directed against the protein residues on the lenses rather than the lens polymers or lens systems care or bacteria. Interestingly, although the inflammation is similar to VKC the recent literature differentiates its pathogenesis from GPC. A continuous mechanical stimulus and an abnormal immunological response are mainly responsible for this disease (135). Contact lenses, ocular prostheses, exposed sutures, elevated corneal deposits and extruded scleral buckles are able to induce conjunctival micro-trauma and corneal irritation leading to inflammatory mediators release. It is suggested that released IL-8 leads to dendritic cell recruitment and enhances antigen presentation to T cells (195,196). Furthermore, altered tear clearance leads to conjunctival epithelial changes and concentration of inflammatory mediators with antigen adhesion (197). Histological and cytological features may be similar with VKC. Mast cells and lymphocytes are increased but eotaxin eosinophil recruitment and activation does not seem to have a major role in GPC immunopathogenesis (198-200) since eotaxin levels do not seem to be increased in GPC patients. Despite the similar histological features with VKC, there are differences in the tear profiles. However, IL-6 and MMP-2 were the only two factors significantly increased both in VKC and GPC compared to normal tears and it is suggested that they may participate in papillae formation (199).

In the conjunctival area of allergic inflammation, using *in situ* hybridization, it was reported an increase in mRNA expression of IL-3 and IL-4 in T cells, reflecting a Th2 pattern in GPC (159). T cell lines from contact lens induced GPC produce low levels of cytokines compared to VKC and AKC (198). Additionally IL-5 was reported to be localized to eosinophils in GPC conjunctival biopsies with antigen expression and cytokine production in chronic allergic diseases of the eye (154). Another study showed that IL-6, IL-8, RANTES and TNF α were expressed in the conjunctival epithelium and IL-8, which chemoattracts neutrophils and eosinophils, was increased in GPC (160).

There are histopathological similarities with VKC (202,203). More specifically the conjunctival epithelium appears thickened and irregular overlying the giant papillae with invaginations into the stroma. The papillae contain epithelial cells, goblet cells, mucus granules, leucocytes, mast cells, lymphocytes, eosinophils, basophils, neutrophils and newly formed vessels among fibrosis (200,203-205). Normal individuals do not have mast cells in their conjunctival epithelium and the presence of these cells as well as eosinophils, basophils in the epithelium or the stroma reflect the allergic inflammatory reaction in GPC (206). The reversible nature of this condition upon removal of the biomaterials inducing the pathological changes may suggest that remodelling is not as severe as seen in VKC, or AKC. No study to date investigated the expression of remodelling markers in GPC and compared them to the other forms of chronic ocular allergy.

Eosinophil infiltration is pathognomonic for ocular allergic diseases. In previous studies eosinophils were detected in the conjunctiva in biopsies from contact lens induced GPC (203,204) and ocular prosthesis GPC (204,205). It has been reported that there is a significant eosinophil infiltrate in GPC tissues which was even higher than VKC or AKC (154). Eosinophils were recruited mainly in the stroma rather than the epithelium but there was no correlation between the cellular response of cytokines and degree of symptoms (208). T cells were also found to be increased. Lymphocytes are found in the normal conjunctiva but reported to be increased (both CD4+ and CD8+ T cells) in GPC by means of immunohistochemistry studies (154,209).

1.8 Classification of Ocular Allergic Diseases

Two major classification systems provide a useful framework to differentiate between the various forms of ocular allergic disease. They are based on the clinical features, immunopathogenesis and duration of disease. Therefore classification systems can be dependent on new evidence and the advances in the understanding of aspects that are being looked up meaning that there is a dynamic situation where criteria can change. Multiple mediators, cytokines, receptors, growth factors and possibly other not yet known processes lead to the development of different clinical signs and symptoms and are differently expressed in the various forms of ocular allergy.

Ocular allergy forms range in severity from mild to severe which may lead to visual impairment. In SAC or PAC the cornea is not involved whereas in AKC or VKC there is corneal involvement and structural as well as functional conjunctival changes related to the inflammatory effects of the cellular and biochemical mechanisms involved.

The European Academy of Asthma, Allergiology and Clinical Immunology (EAACI) Classification (210).

Based on this classification an immunopathological approach is suggested for allergic conjunctivitis which divides it into two broad categories: IgE mediated and non-IgE mediated conjunctivitis (see later section on pathogenesis for detailed description). IgE mediated conjunctivitis can be divided into intermittent and persistent allergic conjunctivitis and persistent allergic conjunctivitis into AKC and VKC. This system has some limitations: GPC is a non-IgE mediated form of ocular allergy and does share some common features with VKC. However, this classification system does not consider all types of allergy.

The International Ocular Inflammation Society (IOIS) Classification 2006 (211).

This is more comprehensive approach and proposed that allergic conjunctivitis refers to a collection of hypersensitivity disorders that affect the lid, conjunctiva and/or cornea. More specifically, it proposes that allergic conjunctivitis is non- infectious immune mediated and can be IgE or non-IgE mediated. Various forms are included based on the traditional classification of ocular allergy: SAC, PAC, VKC, AKC, GPC and drug-induced dermatconjunctivitis. The external eye represents an ideal site of immediate hypersensitivity reactions because of the high number of mast cells in the conjunctiva and the potential for local IgE production. The diseases range in severity from mild to severe forms and all can potentially interfere with the quality of life and lead to visual impairment.

In the 1980s it was initially thought that all types (SAC, PAC, VKC and AKC) are IgE mediated. A grading system for VKC was proposed in 2007 and suggested treatment options for each grade based on clinical signs and symptoms of ocular surface inflammation. This study was the first to describe a new grading system of vernal keratoconjunctivitis that may help clinicians and researchers to classify disease activity and to establish a common agreement for treatments (212). Similarly a grading system for AKC was also proposed that was based on clinical severity taking into account symptoms and signs (213). An overview of the classification is shown in Table 1.4. This thesis will attempt to make new suggestions based on the results in chapters 3 and 4 and enhance our understanding of the immunopathogenesis of the severe forms of ocular allergy and the possible role of growth factors and epithelial remodelling in disease re-classification.

	IgE MEDIATED	IgE and non-IgE MEDIATED	Non-IgE MEDIATED
INTERMITTENT	<i>SAC</i>		
PERSISTENT	<i>PAC</i>	<i>VKC</i>	<i>GPG</i>
CHRONIC		<i>AKC</i>	<i>CDC</i>

Table 1.4: Types of Ocular Allergic disease and their classification (132).

1.9. Clinical Characteristics and forms of ocular allergy

1.9.1 Seasonal and Perennial Allergic conjunctivitis

SAC is the most common allergic ocular condition and is usually caused by pollen and the symptomatology is limited to the season (commonest in spring and autumn). The classic symptom of allergy is itching. Vasodilation leads to an erythematous appearance and chemosis may be present (Figure 1.14). The vasodilation is more superficial and pink rather than deeper and red. The chemosis may be subtle and thus only visible on slit lamp examination. The lids may also be swollen which peaks at 15-30 minutes after exposure and dissipates slowly (10).

There may be eyelid swelling and it could be transient. The intensity of the symptoms is proportionate of the allergen exposure. Clinical history may include atopy, allergic rhinitis and asthma. Allergens drain to the nose and may contribute to nasal involvement. Evidence for this eye to nose drainage pathway is described in the conjunctival allergen challenge model (10,214-215). This is also confirmed by the reduction of nasal symptoms after instillation of ocular therapy (214,215). These disorders may resolve either spontaneously upon removal of the allergen or due to treatment. They are self-limiting and not associated with corneal involvement or visual impairment. PAC is caused by more persistent allergens such as cat dander and dust mites leading to a more chronic form of inflammation. The symptoms are similar to SAC and have a significant effect on the patient's quality of life, health and comfort.

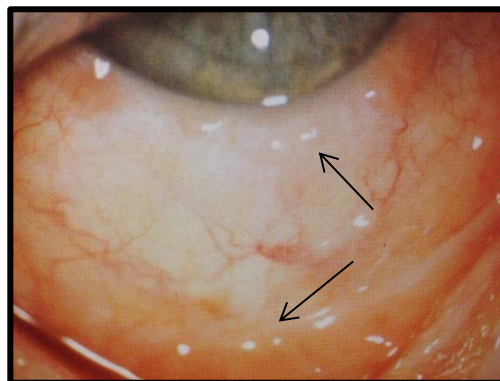


Figure 1.14: Seasonal allergic conjunctivitis with chemosis (arrow) (216).

1.9.2. Vernal Keratoconjunctivitis

VKC is a chronic allergic disease with seasonal exacerbations and characterized by severe itching, tearing, mucous secretions, foreign body sensation and photophobia. (10,217,218). This is a rarer (less than 1% affected) sight threatening disease in patient with an atopic history and an onset before the age of 10 years (218-220). It can resolve during puberty and can last for up to 10 years although in some cases it may still be present in adult age (10,217,218,221).

Usually it affects young males in dry warm climates e.g. Mediterranean countries, West Africa. 75% of patients have a family history of atopy and patients can have a significant history of atopic diseases e.g. eczema, asthma. Clinical features may be asymmetrical and not always bilateral and include superior cobblestone papillae (Figure 1.15), limbal gelatinous nodules, Trantas limbal dots and a Shield ulcer. There could be corneal involvement from superficial punctate keratitis to ulceration and shield formation leading to scarring and visual impairment (10,221,222). Other features may include keratoconus, astigmatism, neovascularization and steroid induced complications such as cataract and glaucoma (10,222). There is a chronic low-level inflammation all year round with a seasonal worsening. The intervals between intense symptoms' periods and symptom-free periods are variable which makes the management of the disease challenging and needs adjustment in each patient individually.



Figure 1.15: Giant papillae in VKC (223)

The clinical diagnosis of VKC is based on history and clinical examination. Important points in history include: (1) the age of the patient, (2) positive atopic family history, (3) association with allergic conditions in the present and the past. It rarely presents before the 3rd year or after the 25th year of life. It was observed that the mean age of the patients was 11+/- 6 years (range 2-32) (218). Positive family atopic history was present in 48.7% with asthma being the most common atopic disease present. VKC was the only manifestation in 58% (218).

Negative prognostic clinical factors include: (1) the size of giant papillae which are related to the persistence of the disease or worsening of symptoms in the long-term period. (2) the bulbar form of the disease which is worse long-term than the tarsal form (224). Corneal ulcers appear in 10% of cases and steroid induced cataract and glaucoma in 2% (224). The reasons for mysterious spontaneous resolution of the disease after puberty are still not fully understood but it is thought that hormonal changes may play a role.

1.9.3. Atopic keratoconjunctivitis

This form of chronic ocular allergic disease affects usually males and females aged 30-50 years. There could be a family history of allergy, asthma, atopy, eczema, urticaria. Typically patients have atopy and either atopic dermatitis or asthma. 3% of the population has atopic dermatitis and approximately 15-40% of those will develop ocular disease. Symptoms include bilateral severe itching, tearing, burning and photophobia. Currently there is no agreement about the diagnostic criteria for AKC. Unlike VKC, AKC is bilateral and symmetrical.

Eyelids can be involved and may be swollen with a scaly appearance and patients may suffer from lid eczema. Blepharitis, meibomian gland dysfunction and dry eyes may also be present (atopic blepharoconjunctivitis) leading to a thickened lid margin. The conjunctiva is hyperaemic and chemotic with tarsal conjunctival papillae seen usually in the early years of the disease. Later in the course of the disease the papillae may be replaced by sheet scarring with or without shortening of the fornices. Gelatinous nodules may be present with or without Trantas dots which occur usually during exacerbations due to limbal tissue hypertrophy. Corneal involvement in the form of filamentary keratitis and punctate erosions may progress to frank corneal erosions and Shield ulcer with a mucous plaque development within 24 hours. These changes may lead to scarring, corneal thinning, corneal vascularization, perforation and sight impairment. Atopic keratoconus and cataract can also be seen in these patients. Posterior subcapsular cataract and glaucoma may also develop secondarily to the prolonged steroid use. *S. aureus* may complicate further the picture causing bacterial conjunctivitis and keratitis as well as herpes simplex virus making difficult to differentiate between acute herpetic and acute atopic disease causing a corneal plaque.

As mentioned above there is no agreed definition for AKC to date. There have been attempts but either did not take into account that patients may not suffer from atopic dermatitis but be positive for other atopic diseases or ignore the corneal involvement. A recent proposal attempted to define the disease is as follows:

“A chronic ocular surface noninfectious inflammatory condition with the following features:

- (1) Always associated with atopic conditions (typically atopic dermatitis, periorcular eczema, or asthma)
- (2) Occurring at any time point in the course of the associated atopic disease and independent of its degree of severity
- (3) With evidence of corneal involvement at some time in the course of the disease”. (225)

1.9.4 Giant papillary conjunctivitis

GPC is a chronic hypersensitivity inflammation of the conjunctiva with the formation of giant papillae (> 0.3 mm) on the tarsal conjunctiva due to mechanical trauma in the presence of predisposition to allergy (Figure 1.16). More specifically it is associated with contact lenses, ocular prosthetic implants, scleral buckles or sutures in contact with the conjunctiva. Prolonged contact lens wear eg patients who sleep with disposable lenses are three times more likely to develop GPC. Clinical features include, itching, tearing, mucous secretions, foreign body sensation, lens intolerance which may necessitate discontinuation and blurred vision. The disease can occur with any type of contact lens material. In contrast to VKC and AKC, the disease is completely reversible upon removal of the foreign body and the cornea is not affected.



Figure 1.16: Upper tarsal conjunctival giant papillae in GPC (10).

1.9.5 Treatment strategies in Ocular allergy

Initially, patients are advised to avoid allergen exposure. They can also be advised that cold compresses may be helpful in symptom relief and the use of lubricants may help in diluting the allergens-antigens in the ocular surface. Topical antihistamines such as pheniramine (first generation) are available over the counter combined with vasoconstrictors. Second generation selective H1 antagonists such as levocabastine are more effective and shown to reduce itching in SAC and PAC but their benefits are short term (226). Systemic antihistamines are commonly used to control allergic rhinoconjunctivitis but also in more severe cases such as AKC and VKC, as an adjuvant therapy to anti-inflammatories. Mast cell stabilizers (sodium chromoglycate, lodoxamide) have an unclear mechanism of action but lead to decreased histamine degranulation. Therefore they may need a few days before their effect takes place and they can be combined with other drugs if an immediate symptomatic relief is required. Dual-acting agents combine mast cell stabilizers and antihistamines and form the first-line drug for many patients.

Non-steroidal Anti-inflammatory drugs such as ketorolac can be used to alleviate ocular pruritus but is not as effective as antihistamines (226). Topical steroids are very effective but may cause significant side effects such as cataract and raised intraocular pressure. Therefore it is recommended that topical steroids are used in short term periods during acute flare-ups. Preservative free dexamethasone 0.1% or prednisolone 1% are preferred and are equally potent. Fluorometholone has poor intraocular penetration and is therefore is a good option for ocular surface inflammatory disease but is less efficacious. Steroid superior tarsal injections can be also used in cases with corneal or limbal involvement not responding conventional therapy. Giant papillary conjunctivitis can resolve upon removal of the biomaterial and symptomatic control can be achieved with artificial tears and control of the inflammatory response with topical steroids.

Topical cyclosporine A (2%) has been shown to be effective in the management of AKC and VKC (227) especially as a steroid sparing agent to prevent their long term use side effects. it was recommended that the minimum dose for the management of shield ulcers is 1% (227). Because T cells are central in the pathogenesis of these

diseases, as described above, use of topical cyclosporin A can offer beneficial effects with important changes in the conjunctival immune cell profile. The *in vitro* effects of cyclosporine A translate into a reduction of T cells and decrease of T cell activation as well as T cell cytokine expression (228). This is described in greater detail in the following section on the role of the epithelium in allergic eye disease. There are also a small number of papers advocating the oral administration of cyclosporin A in severe AKC (229).

Finally, surgical options include excision or cryotherapy of giant papillae may help in resolution of epitheliopathy or corneal ulcer but papillae can regrow. Surgical debridement of the ulcer may be required if resistant to medical treatment and amniotic membrane transplantation is an option in the case of persistent epithelial defects. In steroid induced glaucoma the patient may require trabeculectomy surgery.

Once remodelling is established with permanent tissue structural, functional changes, disease perpetuation and exacerbations, achieving disease control is harder. Similar observations are seen in chronic asthma. As explained before a re-classification of ocular allergic disease and consensus are required based on the clinical signs to decide therapy. Proactive management is often advised before seasonal exacerbations with environmental modifications and systemic medications. Therefore the need of new therapeutic strategies aiming to offer better symptomatic relief and arrest of the remodelling changes at the molecular and cellular level, are suggested as new therapeutic options to explore.

1.9.6 Chronic “autoimmune” conjunctival inflammatory disease: ocular cicatricial pemphigoid

Ocular cicatricial pemphigoid (OCP) (now referred to as ocular mucus membrane pemphigoid) is a systemic autoimmune vesiculobullous disease directed against mucosal basement membrane, affecting mucous membranes, oro-pharyngeal mucosa (85% of cases), the skin (25% of cases) and the conjunctiva (65% of cases) (230). Vesiculobullous eruptions occur with a chronic course and acute inflammatory episodes. The lesions heal with scarring and the mucosal inflammation leads to cicatrization. Chronic progressive inflammation of the conjunctiva leads to subepithelial fibrosis, symblepharon, lid abnormalities, sicca syndrome and distichiasis. Corneal involvement may occur from direct epithelial damage and abnormal tear film production. The conjunctiva shortens due to fibrosis following episodes of inflammation. In OCP, most patients have a progressive form of bilateral conjunctival disease and some may develop blindness within months from disease onset.

The autoimmune nature of OCP involves immunoglobulins and complement at the conjunctival basement membrane, autoantibodies in serum, T cell cytokine dysregulation and response to immunosuppression. OCP is probably an autoimmune disease (“autoimmune conjunctivitis”) with a chronic progression and can lead to blindness. Its severe chronic nature with fibrosis and corneal involvement may suggest that remodelling plays a role in its pathogenesis. Approximately 75% of patients require systemic immunosuppression (231) and 45% require continuing systemic treatment to avoid reactivations (232). Most patients present during the fibrotic stage of the disease and cicatrization may progress despite immunosuppressive treatment (233). Various immunosuppressants are used including mycophenolate mofetil which seems to be effective and well tolerated for moderately active OCP (230). Others used include, dapsone, azathioprine, methotrexate and cyclophosphamide with prednisolone. Over the last decade significant advances have been aiming to improve our understanding of the pathological mechanisms involved in the fibrosis in pemphigoid disease affecting various tissues. However the conjunctival fibrosis in OCP has not been studied in similar depth yet.

1.10 Summary

Allergic ocular disease is common with increasing prevalence and affects more than ten million patients in the UK. The spectrum of ocular allergy spans across the mild and reversible forms to the other end of irreversible and chronic sight-threatening diseases. The conjunctival mucosal membrane plays a crucial role in the immunopathology of ocular allergy and it has been proposed that its structural and functional alterations in allergic disease may contribute to disease severity and progression. Although previously the majority of research work was based on the fibroblast and subepithelial areas of the conjunctiva, recently there has been more attention drawn to the roles of the epithelium.

Over the last 8 years there has been more progress in understanding the immunopathogenesis and clinical behaviour of allergic eye disease based on direct clinical evaluation, histological assessment and *in vitro* studies. It has been suggested that IgE hypersensitivity, cytokines and T cells on their own may not be enough to account for disease severity and perpetuation. Other local factors have been thought to co-participate such as remodelling of the conjunctival tissue. Remodelling of the epithelial cells leads to permanent changes in the local cytological architecture and functional deficiencies. VKC has been the form of ocular allergic disease mostly studied. Drs Leonardi and Bonini from Italy, have demonstrated that remodelling markers can be overexpressed in VKC and have attempted to produce diagnostic and treatment recommendations for this disease. Similarly Dr Calogne's team in Spain suggested a grading system for AKC. However, the need of a general consensus for an improved classification system of ocular allergy, based on clinical signs and therapeutic decisions, remains a major task to be achieved.

So far there is published work that investigated the distribution of collagens and growth factors mainly in VKC but no reason has been proposed as to why this occurs making AKC and VKC different to the other forms. An important claim of this study is that remodelling constitutes an important immunopathological feature that distinguishes the reversible from the persistent and chronic forms of allergic conjunctivitis.

In addition no much is known about the role of remodelling in the immunopathology of GPC. There is no study to date to compare the detection of epithelial remodelling markers in GPC and the other chronic or persistent forms of allergic conjunctivitis.

The presence of eosinophils and T cells in the conjunctival tissue, as seen in VKC and AKC, in biopsies of patients with GPC may suggest that remodelling molecules may be detected. Given the reversible nature of the condition one could expect that the expression of remodelling molecules, if any, would not be at the levels seen in the severe forms of ocular allergy.

1.11 Aims and Hypothesis

The conjunctiva being a mucosal membrane shares many common features with the respiratory tract where the mucosa is also directly exposed to environmental factors. Based on the recent epithelial-mesenchymal trophic unit hypothesis in asthma it is suggested that a similar hypothesis may be applied in the chronic forms of ocular allergy. The central aim of this thesis is to investigate potential candidate remodelling epithelial molecules of the conjunctiva, involved in the EMTU hypothesis, in reversible and irreversible forms of ocular allergy.

Chronic inflammation is a feature of chronic or persistent ocular allergic disease but can also be seen in other disease such as ocular cicatricial pemphigoid, also known as “autoimmune conjunctivitis”. Therefore the following question is set: Can epithelial remodelling markers of the EMTU hypothesis be detected in other forms of chronic conjunctival inflammation of immunological origin? The line of argumentation hinges on the claim that the EMTU hypothesis may be applied to chronic mucosal inflammatory epitheliopathy of allergic immunopathological nature rather than autoimmune as seen in OCP.

As seen in chronic asthma, Th1 and Th2 cytokine participation is not enough to account for disease severity. It is thus hypothesised that increased conjunctival epithelial growth factors related to remodelling contribute to abnormal epithelial repair in the chronic forms of disease as opposed to reversible forms without corneal involvement. The methodology in Chapter 2 of the thesis consists of an initial investigation to look for evidence of remodelling markers associated to the EMTU hypothesis in conjunctival tissues from patients with ocular allergy, and further *in vitro* experiments to explore the behavior of the conjunctival epithelial cells under inflammatory conditions in relation to those remodelling markers. Employing immunohistochemistry, *in vitro* cell line cultures, cell viability assessments and ELISA assays, my studies were designed to investigate the following:

- 1) The expression patterns of EGFR, its ligand TGF α , and CD44 in chronic forms of ocular allergic disease (AKC/VKC) compared to reversible forms of ocular allergy (SAC/GPC and normal non-inflamed subjects) by means of immunohistochemistry.
- 2) The detection, if any, of EMTU associated molecules in other types of chronic inflammatory conjunctival disease. To address this, conjunctival biopsies of chronic uninflamed and actively inflamed ocular cicatricial pemphigoid are used.
- 3) The *in vitro* secretion of EGFR, TGF α , sCD44, p21^{waf} and VEGF-A by conjunctival epithelial cells in response to treatment with various cytokines. It is aimed that human conjunctival epithelial cell lines can be used to develop an *in vitro* model of epithelial remodelling molecules in ocular inflammation.
- 4) T cells are known to participate in the immunopathogenesis of chronic ocular allergic disease and given the recent identification of the new subset of Th response Th-17, and the suggested role of IL-17E (or IL-25) in remodelling in asthma, this thesis will also investigate the effect of IL-17A and IL-25 on remodelling conjunctival epithelial markers.
- 5) Finally, an attempt is made to apply the results of the study aiming to enhance our understanding of the immunopathogenesis, classification and new therapeutic strategies of ocular allergic disease.

CHAPTER 2

MATERIALS & TECHNIQUES

2.1 Conjunctival biopsies

Conjunctival biopsy tissue sections used throughout this study were obtained from our Tissue Bank, as part of our immunological studies in conjunctival allergic mechanisms.

2.1.1. Biopsy of non-inflamed subjects

The conjunctival biopsy specimens were originally harvested prior to the commencement of cataract or strabismus surgery from non-atopic individuals, non-age matched. Patients undergoing elective surgery were asked to participate in the study and if fulfilling any of the following criteria were excluded (Research & Development Ethics Approval Reference number: LIGS1012, M Hingorani's MD Thesis, University of London, 2000):

1. Age <18 years
2. External eye disease
3. Concurrent systemic treatment that may influence the immunological response (e.g. leukemia, steroids)
4. Concurrent topical ocular treatment (steroids within 1 month)
5. AIDS/ Hepatitis B.

2.1.2 SAC biopsies.

The specimens were originally harvested prior to the commencement of a randomized trial and sections prepared for immune studies. For the SAC group, two biopsies were included in the group of controls. The SAC patients were challenged with allergen 24 hours before biopsy procedure. Timothy plus rye grass combination was used and a reported positive response was documented prior inclusion. Patients with a history of SAC were challenged as part of a phase II clinical trial (Reference number CAT-213-0203) and were all consented to donate tissue biopsies with Ethics approval.

2.1.3 Biopsies of patients with GPC, VKC, AKC:

Conjunctival biopsies were obtained by consented patients according to the regulations of the Research and Ethics Committee of Moorfields Eye Hospital (Research & Development Reference Number LIGS1012). They were subsequently stored -80°C and used exclusively for research purposes.

Patients with active disease and symptoms were recruited from the External eye disease clinics of Moorfields Eye Hospital and any of the following features excluded them from the study (M Hingorani's MD thesis, University of London, 2000):

1. Age <18 years, except in the case of VKC giant papillae between 11-18 which might have been biopsied with the child's and parents' informed consent.
2. Other external eye diseases
3. Concurrent topical ocular therapy (steroids within 4 weeks prior to biopsy and sodium chromoglycate within 7 days prior to biopsy)
4. Concurrent systemic disease or therapy that might have influenced the immunological response (e.g. leukemia or steroids)
5. AIDS/Hepatitis B.

The diagnoses were made clinically and all AKC patients also suffered from atopic dermatitis. All AKC and VKC patients had current or previous corneal involvement. The age of the VKC patients was higher than expected since ethical approval of this study required patients to be over 16 years old.

2.1.4 Biopsies from OCP patients.

Initially approval by Research and Ethics Committee of Moorfields Eye Hospital was obtained (Reference number 05/Q1604/126) and patients with chronic uninfamed OCP, actively inflamed OCP and nomal uninfamed subjects were consented to donate conjunctival biopsies for research purposes (Dr V Saw PhD Thesis, University of London, 2009).

2.1.5 Antibody optimization studies

Initially preliminary immunohistochemistry studies took place to determine suitable antibody concentrations for the study for EGFR, its ligand TGF α and CD44 acting as a positive control. Initially sections of tissue were selected for the purpose of titrating each antibody, to determine the optimal dilution for staining. The final dilution selected was then used throughout the study. Optimal conditions and dilutions of all antibodies were determined in the initial experiments with various dilutions and variable antibody incubation times.

Cytokeratin18 antibody, a known epithelial cell marker, was used with the antibodies under investigation for overnight double staining immunohistochemistry under various antibody dilutions to assess similarities and differences in the cell expression and distribution. Cytokeratins (CKs) are a heterogeneous class of approximately 30 structurally related polypeptides that comprise the intermediate filament system characteristic of epithelial cells. The expression of cytokeratins in the normal human conjunctiva has been investigated (228). Cytokeratin 18 is known to be detected in epithelia and has been chosen for this preliminary study in an attempt to localise the conjunctival epithelium and assess if the remodelling epithelial markers can be co-expressed in similar epithelial regions of the tissue (228).

Initially random biopsies from different subjects from the controls and the CAED groups were selected, stained and analysed for various antibody concentrations and graded by a masked observer. Single antibody overnight staining was used for the following antibodies shown in Table 2.1 (for results see chapter 3).

Monoclonal mouse, anti human TGFα	Clone MF9+TG86 Abcam, UK
Monoclonal mouse, anti human CD44	Clone DF1485 Dako, Denmark
Monoclonal mouse, anti human EGFR	Clone E30 Dako, Denmark
Monoclonal mouse, anti human Cytokeratin 18	Clone CY-90 Sigma, USA.
Monoclonal goat Control IgG	R&D systems, UK.
Polyclonal Rabbit F(ab) ₂ (1:200)	Dako, Denmark

Table 2.1: Antibodies used for optimization study

2.1.6 Ocular allergy and OCP groups participating in study

As described above, conjunctival biopsy specimens from a tissue bank maintained at the Institute of Ophthalmology were obtained from normal conjunctival tissue, seasonal allergic conjunctivitis (SAC), Giant papillary conjunctivitis (GPC), vernal and atopic keratoconjunctivitis (VKC, AKC) were divided into 2 groups. VKC and AKC are severe and chronic forms of ocular allergy that may involve the cornea whereas SAC and GPC not.

Controls group: consisting of normal non-inflammatory subjects (n =6) GPC (n=3), SAC (n=2) biopsies. All patients had active disease without corneal involvement. GPC and SAC are reversible forms of ocular allergy.

Chronic Allergic Eye Disease Group (CAED): severe and chronic disease group consisting of VKC biopsies (n=4) and AKC (n=4) biopsies (total n=8).

Ocular Cicatricial Pemphigoid (OCP) Biopsies:

- Group1: chronic uninflamed (n=6)
- Group 2: active inflammation (n=9)
- Group 3: normal conjunctival biopsies (n=7)

Details of all ocular allergy and OCP patients who participated in the study can be found in Appendix 1 and the final antibody dilutions used in the study are shown below in Table 2.2:

Monoclonal mouse, anti human TGFα	Clone MF9+TG86, Abcam, UK	1:100
Monoclonal mouse, anti human CD44	Clone DF1485, Dako, Denmark	1:200
Monoclonal mouse, anti human EGFR	Clone E30, Dako, Denmark	1:200
Monoclonal goat Control IgG	R&D systems, UK	1:100

Table 2.2: Final antibody dilutions in the study.

2.1.7 Immunohistochemistry principles and techniques

Immunoperoxidase procedures allow visualization of cell components in frozen tissue sections. These methods involve the use of antibodies and the enzyme peroxidase and utilize an enzyme-substrate reaction to convert colourless chromogens into coloured products. A commonly used enzyme is horseradish peroxidase (HRP) which is small in size and does not interfere with the binding of antibodies to adjacent sites. It forms a complex with the substrate hydrogen peroxide (H_2O_2) which then oxidizes an electron donor to produce the end product of the reaction which is a coloured molecule and water. 3-amino-9-ethylcarbazole (AEC) is a chromogen or electron donor which can be oxidized to become a coloured end product (forms a red product upon oxidation). The end product is alcohol soluble and therefore must not be stored immersed in alcoholic solutions. Instead an aqueous counterstain and mounting medium are used.

The staining method utilizes an avidin or streptavidin biotin-peroxidase complex (ABC method) which is a more sensitive method than the direct, indirect and PAP/APAAP procedures. The ABC technique utilizes the high affinity avidin glycoprotein to the vitamin biotin where a complex of peroxidase conjugated biotin and avidin is formed. The free sites of the avidin molecule allow binding to biotin present on a secondary biotinylated antibody which links the complex to the primary antigen-specific antibody. The biotinylated antibody does not have to be added in excess since free F(ab)' sites are not required for binding. The strong affinity of avidin and streptavidin for biotin and a mild biotinylation process of the secondary antibody make these methods more sensitive.

Background staining is a common problem in immunohistochemistry. The commonest reason is the nonspecific linking of the antibody to collagen of the connective tissue. In order to avoid this problem, an innocuous protein (non-immune serum from the same animal species) is added before applying the antibody. Additionally endogenous peroxidase present in the specimens may also contribute to background staining. A common method to suppress peroxidase activity is section incubation in 3% H_2O_2 or a mixture of sodium azide and H_2O_2 . Finally the liability of

Fc receptor proteins present on macrophages and granulocytes may also contribute to non-specific staining. The use of F(ab)₂ fragments may help avoid this problem.

2.1.8 Preparation of Biopsies

Specimens were previously obtained from the upper tarsal conjunctiva anaesthetized with amethocaine 1% and lignocaine 2% with adrenaline. A 3 mm trephine was used to obtain a sample from the central third of the upper tarsal conjunctiva which is the site of maximal inflammatory involvement (168). Where giant papillae were present one or occasionally two were shaved from the surface instead. In addition a small snip of bulbar conjunctiva was taken from the superior fornix.

The specimens were immediately placed in the ice-cooled acetone containing protease inhibitors iodoacetamide (20mM) and phenylmethylsulfonylfluoride (2mM) and stored overnight at -20°C. The specimens were then processed for and embedded in glycerol methacrylate resin.

Cleaned slides were placed into racks and immersed into a freshly prepared solution 2% of APES (3-amino propyl tri-ethoxy silane) adhesive in 99% industrial methylated spirit for 10 seconds. This was followed by a wash in 99% methylated spirit and distilled water for 5 minutes each. Then the slides were left to dry overnight at 37°C.

6 µm thick sections were cut using a cryostat (600 Cryotome, Angla Scientific) at -20°C. Correct orientation was ensured using 0.1% toluidine blue-immersed sections checked under a light microscope and re-orientated if required. Correctly orientated sections were cut and left to air-dry at room temperature for 1-2 hours before storage at -20°C for immunohistochemistry studies.

Plastic embedding media (epoxy resins and methacrylate) provide an attractive alternative to frozen and paraffin-embedded tissue sections. The epoxy resins polymerise evenly and preserve structure details but interact with tissue during this polymerization process and are therefore not suitable for immunohistochemistry studies. Methacrylates do not polymerise with the tissue and prevent alterations of

the antigens. Therefore they are more suitable for immunohistochemistry studies but may cause shrinkage of the tissue specimen and poor morphology.

Prefixation of biopsies was carried out in acetone plus protease inhibitors since it was thought that mucosal biopsies contain proteolytic enzymes which may lead to tissue digestion according to the ModAMex method (modified cold acetone fixation with subsequent methylbenzoate and xylene treatment)

2.1.9 Single antibody Immunohistochemistry

An ultramicrotome was used to cut 2 μm sections which were acetone fixed for 20 minutes and allowed to air dry. Peroxidase block was added for 10 mins and then rinsed in PBS 0.05% 3 times. Foetal Calf Serum (FCS) 10% 150 μl was added for each group for 30 minutes and then wiped out without rinsing. This was followed by addition of 150 μl of antibody in the concentrations stated above. Following addition of the antibodies slides were incubated in humidified chamber in room temperature overnight.

The slides were washed 3 times for 5 mins each with PBS. F(ab)_2 biotin (diluted 1:200 in PBS) was added in each slide and was incubated for 2 hours in room temperature. Furthermore the slides were rinsed in PBS 3 times for 5 minutes each. SA peroxidase diluted 1:300 in PBS was added to the sections and incubated for 2 hours in room temperature and PBS rinsed as described in the step above and further developed for 25 mins with 50 μl amino ethyl carbazole (AEC).

Following this, the sections were rinsed with H_2O in running tap for 5 minutes. For cell counts and background staining of the tissue, the slides were put through into Mayer's haematoxylin for 1 minute and then rinsed with tap water running through the blue. The slides were then covered and mount in glycergel. After immunohistochemistry the cells staining positively for the antibody identification markers were identified by a red deposition of the AEC reaction product.

2.1.10 Immunostaining: grading and analysis

Microscopy examination and quantitative grading assessment was carried out with four x40 adjacent fields per section per antibody which were examined by a masked observer to include both epithelial layer and stroma with an MOTIC BA400 microscope. The intensity of the antibody uptake was graded for each field which was scored separately for the epithelium and the stroma. Then an average score was taken for the four fields of each section for the epithelium and stroma and finally the scores were grouped for each antibody and disease group.

In order to quantify staining a simple scoring system was used as illustrated in Table 2.3 below as used in similar studies (127,128). Dot plots of the data were developed and were analyzed using Mann-Whitney U-test (two-tailed) non-parametric statistical analysis to compare the grading scales. A p value <0.001 was taken as highly statistically significant. Examples of the degree of staining expected for each score are shown below using randomized conjunctival biopsies and shown overleaf in Figures 2.1-2.4. The localization of the staining was also recorded, either within the epithelium or the stromal areas in each section. Where a small cluster or isolated cells expressed the antibody, this was graded as 0.5+.

Score	Antibody detection grade
0	no staining
1+	mild uptake (few cells positive per field)
2+	moderate uptake (pink uniform staining)
3+	intense uptake (intense red uniform staining)

Table 2.3: Grading system for biopsy sections

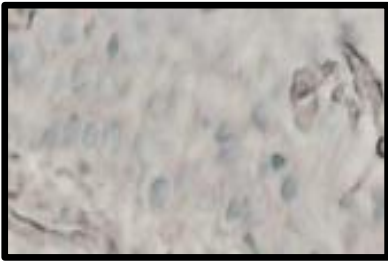


Figure 2.1: Negative control showing background staining of the epithelium.



Figure 2.2: Example of epithelial mild (1+) antibody staining (arrow).

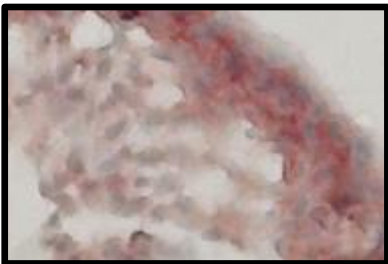


Figure 2.3 Example of epithelial moderate (2+) of antibody staining.

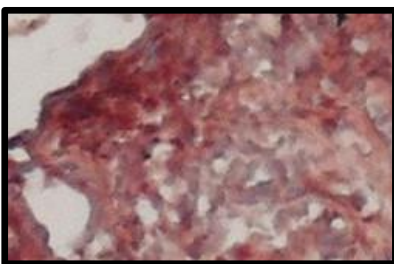


Figure 2.4: Example of epithelial intense (3+) antibody staining.

2.2 *In vitro* studies

2.2.1 Conjunctival Epithelial cell lines

Two different human conjunctival epithelial cell line cultures were used to develop an *in vitro* model to investigate the secretion profiles of EGFR, VEGF-A and sCD44 secondary to treatment with various cytokines. IOBA-NHC (kindly donated by M Calonge from IOBA, University of Valladolid, Spain) and Wong-Kilbourne derivative of Chang conjunctiva ChWK (clone 1-5c-4 ECACC, Idminton, UK) were cultured as described below and assayed in various stimulatory conditions. The culture techniques were as previously described (189,228,229).

These two cell lines were selected because they are the only two non-transfected cell lines and have been previously used to investigate the effects of preservatives, toxicity, allergic and cytokine responses (189, 230-236). Both cell lines share important morphological and functional characteristics compared to primary human conjunctival epithelial cells. To our knowledge no comparison or *in vitro* model of remodelling factors of both IOBA-NHC and CHwK has been performed. Therefore the purpose of this experiment is to determine whether both cell lines are suitable.

2.2.1.1 *In vitro* culture of IOBA-NHC line

Cell line cells were taken from frozen stock (maintained in liquid nitrogen) and incubated at 37 °C, 5% CO₂ in 5 ml medium with DMEM Nutrient mixture F-12 HAM (Sigma-Aldrich) with 10% heat inactivated foetal calf serum FCS (Bio Sera, UK), 100U/ml Penicillin/streptomycin (Invitrogen UK), 2ng/ml mouse EGF (Sigma), 1µg/ml bovine insulin (Sigma-Aldrich), hydrocortisone (5 µg/ml) and 0.1µg/ml cholera toxin (Sigma-Aldrich) in a 25 cm² tissue culture flask.

Cells were plated in 24-well plates and grown until confluent (24-48 hours). Prior to assay, they were maintained for 24 hours in serum-free non-supplemented medium to rest. When confluence was reached cells were transferred to a 25 cm² flask in 10ml IOBA medium and incubated at 37 °C, 5% CO₂. Cells were split and fed when monolayers reached 70-80% confluency.

Furthermore cells were detached with trypsin-EDTA (Invitrogen) and incubated at 37 °C, 5% CO₂ for 3 minutes and then centrifuged at 400rcf for 10 minutes. Detached cells were re-suspended and added to the appropriate plate and incubated at 37 °C, 5% CO₂. Cells were counted using trypan blue exclusion and 50,000 cells /ml plated into 24-well plate (1ml/well).

The cells were grown in IOBA medium and, prior to cytokine treatment, the medium was replaced with serum-free supplemented medium for 24 hours before treating the cells with the cytokines of choice. Control cells received no treatments. After treatment the culture medium was collected, centrifuged and harvested cell-free supernatants were stored at -80°C until assayed. Details of the techniques are described below:

2.2.1.2 Defrosting cells

A solution containing 44.5ml culture medium, 100U/ml P/S and 5 ml FCS under hood is prepared. Cells were taken out of liquid nitrogen and buffered with FCS and DMSO (to maintain cell wall integrity). Cells (5×10^6 /mL) were placed in the incubator for 1-2 minutes to thaw and then mixed immediately with 10 ml culture solution to dilute the toxic effects of the DMSO and centrifuged at 400 rcf for 10 minutes. After centrifuging the cells were re-suspended and 10 ml culture solution added and placed in incubator 37°C, 5% CO₂ for 48 hours.

2.2.1.3 Passaging cells

Upon reaching confluence, the culture medium prepared for the cells is placed in a water bath. Then the medium is removed from the flask and cells are adherent on plastic surface. The flask was then washed with PBS to remove FCS and allow trypsin to mobilise cells from the adherent areas. 5 ml of trypsin was added to 45 ml of PBS and 4 ml of this solution was added into the flask and placed in the incubator for 3 minutes. The same amount of 4ml 10% FCS with culture medium of cell line was added and then centrifuged at 400 rcf for 10 minutes. Then 1 ml of culture

medium was added to re-suspend and a further 10-20 ml to mix within the pipette. Furthermore 10 ml was added in flasks.

At a later stage the fluid was removed from the flasks using a 10ml pipette and cleaned with sterile PBS. 4ml trypsin was added in the flask and placed in the incubator for 3 minutes. 6 ml of medium was added in each flask and then placed in an empty tube and centrifuged at 400 rcf for 10 minutes. After this step the cells were re-suspended with 1 ml medium and 4 ml were added.

2.2.1.4 Cell density calculations

Cells were harvested in a known volume and 10 μ L loaded to a haemocytometer and examined immediately under a microscope (x10 objective). Cells were counted in the central gridded square (1mm²) and multiplied by 10⁴ to estimate the number of cells per mL. Cell viability was also assessed. 1 mL of Trypan blue 0.4% solution is added to 1 mL of cells. The number of blue stained cells and the total number of cells were counted in each field, counting at least 100 cells in total.

The viable cells % was calculated as follows:

$$\% \text{ viable cells} = [1.0 - (\text{number of blue stained cells} / \text{number of total cells})] \times 100$$

To calculate the number of viable cells per mL of culture the following formula was used and corrected for the dilution factor:

Number of viable cells $\times 10^4 \times 2 =$ cells/mL culture. The cell density is the adjusted to aim for 50,000cells/1ml. In every experiment, cell viability was at least 95% prior to use.

2.2.1.5 *In vitro* culture of ChWK line

The cells were taken from frozen stock and maintained in Medium-199 (45ml) with L glutamine (2mM) and 10% FCS (5ml) supplemented with penicillin/streptomycin (100U/ml). As above, cells were seeded at a 50,000cells/1ml density in 24-well plates in culture medium and upon reaching pre-confluence (70-80%) the cells were detached using trypsin-EDTA, centrifuged at 400rcf and split into flasks.

Cells were routinely used between passage #6 and #9 for experiments for both cell lines to ensure there were no changes in function when cells were multiply passaged.

2.2.2 Cytokine treatments of conjunctival epithelial cells.

The epithelial cells were detached as before and centrifuged at 400rcf for 10 minutes. Cells were then re-suspended and plated out in a 24-well plate at 1×10^5 cells/ml and incubated at 5% CO₂, 37 °C. When the cells had reached pre-confluence (70-80%), supernatant was removed and replaced with fresh medium in the presence or absence of various cytokines.

The following Table 2.4 below, shows the types and concentrations of cytokines used to treat the cells at variable time points. The cells were then treated for the time periods as described below. After incubation the supernatants were removed and centrifuged. The cell debris was then removed and cell-free supernatants were frozen at aliquots at -70 °C ready for ELISA assay according to the manufacturer's instructions: BenderMedsystems (sCD44) and R&D (p21, VEGF-A, EGFR, TGF α) protocols.

Cytokines	Final concentrations
TNF α (Peprotech)	25 ng/ml
IL-1 β (R&D systems)	10 ng/ml
LPS (Sigma-Aldrich)	5 μ g/ml
PolyI:C (Sigma-Aldrich)	50 μ g/ml
Grass pollen extract (Sigma-Aldrich)	10 μ g/ml
PMA (Sigma- Aldrich)	1.0 ng/ml
IL-17A (Peprotech)	10 ng/ml
Zymosan (Peprotech)	20 μ g/ml
IL-25 (Peprotech) titrations	1000ng/ml, 100ng/ml, 10ng/ml, 1ng/ml

Table 2.4: Recombinant Cytokine concentrations used to treat cells.

The doses of the stimulatory cytokines used were based on previous work (189). Dose and time response curves were also performed (237). TNF α , IL-1 β , IL-17A, IL-25 are all human recombinant cytokines reported to be expressed in inflammatory responses (237).

Supernatants were taken at various time intervals as specified below. In the steroid treatment study 1 μ M Dexamethasone was added 30 minutes prior to cytokine treatments. All experiments were repeated at least twice and mean values calculated per sample. When combinations of stimulants were selected the concentrations of each were added together.

More specifically fresh culture medium was prepared using the agents described before for each cell line. 250 μ l was placed in each well from the cells tube and 750 μ l (medium with stimulant) were added making a total volume per well of 1 μ l. The following concentrations were used and added as shown below in Table 2.5 in each well.

PMA	1 μ l PMA in 749 μ l culture medium
Zymosan	20 μ l zymosan with 730 μ l culture medium
polyl:C	50 μ l polyl:C with 700 μ l culture medium
LPS	5 μ l LPS with 745 μ l culture medium
Grass	4 μ l grass with 746 μ l culture medium.
IL-17A	1 μ l IL-17 + 2.5 μ l culture medium mixed and 1 μ l taken and placed in well
TNFα/IL-1β	25 μ l TNF α and 1 μ l IL-1 β , with 724 culture medium

Table 2.5: Recombinant cytokine preparation technique

2.2.3 Pilot experiments using IOBA-NHC cells with single and multiple treatments

The initial pilot experiments involved comparing a range of potential stimuli: IL-1 β and TNF α , IL-17A, PMA, LPS, Zymozan, grass pollen extract, poly-IC or culture medium alone as a negative control were used to treat the cells and supernatants were harvested at 24, 48, 72, 98 hours. Cells were treat only once at time point 0. The samples were assayed by ELISA to quantitate secretion levels of EGFR, VEGF, CD44, TGF α , and p21.

Additionally, a combination of treatments was used and sCD44 secretion was assessed by means of ELISA at 24, 48, 72, 96 hours. A greater variety of combinations of treatments was also performed and supernatants harvested at 144 and 192 hours were processed to assess expression of sCD44 and by means of ELISA. Supernatants were harvested by sampling from the same well from every

time point. Additionally, epithelial cell viability and evidence of apoptosis was performed using YoPro staining and flow cytometric analysis.

2.2.4 Single cytokine treatments of IOBA-NHC cells

Preliminary data suggested treatment dependent and time dependent responses by the lines. The next study focuses on single treatment using IL-1 β and TNF α , IL-17A, PMA or culture medium alone as a negative control and supernatants were harvested at 24, 48, 72, 96 hours. EGFR, TGF α and sCD44 levels were assessed by ELISA. p21 detection was also assessed at 144 and 192 hours. Means of duplicate wells were produced for EGFR and p21 and triplicate wells for TGF α and sCD44.

2.2.5 Multiple cytokine treatments of IOBA-NHC cells

PMA, IL-1 β and TNF α , IL-17A or no treatment were used to treat cells at time points: 0, 24, 72 and 144 hours. Before treatment supernatants were harvested and samples processed by means of ELISA for assessment of baseline detection of sCD44, EGFR and VEGF. Harvesting was performed at 24 hours, 72 hours, 144 hours and 192 hours. Means of triplicate wells were developed for VEGF and sCD44 and duplicate wells for EGFR analysis.

2.2.6 Steroid pre-treatment of IOBA-NHC cells and effect of IL-17E (IL-25)

No treatment, or PMA, IL-17A, TNF α /IL-1 β were added to cells at time point 0. Dexamethasone (0.1 μ M, 1.0 μ M, 0.1 μ M, 0.01 μ M, 0.001 μ M) was added 30 minutes prior to cytokine treatment and supernatants harvested at 48 hours. Samples were processed by means of ELISA for EGFR and VEGF.

Further studies were performed with no treatment, IL-25 (1000ng/ml, 100 ng/ml, 10 ng/ml, 1.0 ng/ml), PMA, TNF α and IL-1 β to treat cells and divided in a group without steroid treatment and one group with treatment of 1 μ M dexamethasone. Supernatants harvested at 48 hours were processed by means of ELISA for EGFR and VEGF.

2.2.7 Multiple cytokine treatments of ChWK cells

PMA, IL-1 β and TNF α , IL-17A and no antigen were used to treat cells at time points: 0, 24, 72, and 144 hours. Before treatment supernatants were harvested and samples processed by means of ELISA for assessment of expression of sCD44 and EGFR. Harvesting was performed at 24 hours, 72 hours, 144 hours and 192 hours. (means of duplicates for EGFR and triplicates for sCD44). Additionally lysates were prepared from samples at 144 and 192 hours for p21 expression from duplicate wells that received a single initial cytokine treatment.

2.3 Enzyme-linked immunosorbent assay (ELISA) technique

This quantitative laboratory method was used to detect the specified analytes *in vitro*. The manufacturers' instructions were used for preparation of reagents, test protocol and calculation of results (BenderMedsystems for detecting EGFR, sCD44, VEGF; TGF α , p21 by R&D systems).

A 96-well plate (Immunolon microplate; Nunc,UK) was coated with capture antibody as shown in Table below 2.6 and incubated overnight at room temperature. The medium was aspirated and wells washed 3 times with wash buffer (0.05 % Tween 20, Sigma-Aldrich) in phosphate buffered saline (PBS; Sigma- Aldrich). Plates were then blocked by reagent diluent (1% bovine serum albumin BSA; Sigma-Aldrich) and incubated in room temperature for 1 hour.

The seven point standard curve was made and the highest standard concentrations are shown in the table below. Furthermore wells were aspirated, washed and the detection antibody (as shown in the Table 2.6 overleaf) was added for 2 hours in room temperature. Wells were then aspirated again and washed and Streptavidin-horseradish Peroxidase (HRP) was added to each well and incubated for 20 minutes at room temperature. The wash step was repeated and substrate solution was added to each well and then incubated at room temperature for 30 minutes away from light.

The coloured product is formed in proportion to the amount of the specific protein present in the sample or standard. This reaction was terminated with an acidic stop solution (2N H₂SO₄) and absorbance was measured at given wavelengths depending on manufacturer's instructions (450nm), using an ELISA plate reader (Safire, Tecan). This process is outlined in Figure 2.5 in the next page and may vary for each marker.

For assaying levels of intracellular human total p21, cell lysates were prepared to allow access for the antibodies. According to the manufacturer's instructions for the production of lysates: cells were rinsed with PBS and solubilized at 10,000 cells/ml in Lysis Buffer and after 15 minutes centrifuged at 4000g for 5 minutes. The supernatant was transferred in to a clean test tube and assayed according to the manufacturer's ELISA protocol.

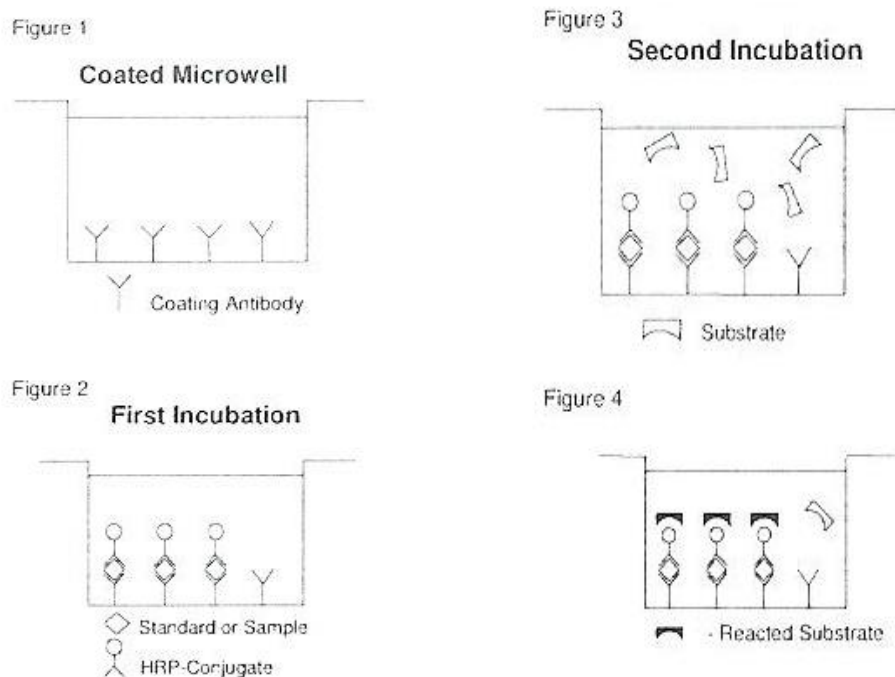


Figure 2.5: Diagram illustrating the principles of the ELISA method (adapted by the product information and manual by Bender Medsystems).

Protein	Conjugate antibody	Standard antibody	Highest standard	Source
p21	180 µg/ml	9 µg/ml	4000 pg/ml	RD systems
TGFα	200 µl from reagent provided	10 ng/ml	1000 pg/ml	RD systems
sCD44	50 µl (1:50 of 10µl provided)	8 ng/ml	4000 pg/ml	Bender medsystems
VEGF-A	50µl(1:100 of 100µl provided)	4 ng/ml	2000 pg/ml	Bender medsystems
EGFR	100 µl as per manufacturer	50 ng/ml	3000 pg/ml	Bender medsystems

Table 2.6: Proteins of interest targeted for detection of ELISA

A standard curve was established for each run since the OD values of the standard curves may vary according to the conditions of the assay performance e.g. operator dependence, washing technique, temperature variations, pipetting technique. Bacterial or fungal contaminations of samples or reagents may cause inaccuracies. Random microscopy of samples in every ELISA assay did not reveal the presence of micro-organisms. Disposable sterile pipettes tips, flasks or sterilised glassware were used and all reusable equipment was rinsed thoroughly to prevent false positive or false negative results. Wells were emptied completely before dispensing fresh wash solution, filled with wash buffer for each wash cycle and did not remain uncovered or dry for extended periods. Frozen samples were thawed once and not reused to prevent loss of the bioactive human analytes under investigation. Stop solution was added uniformly and quickly throughout the microwells to completely inactivate the enzyme according to the manufacturer's instructions for each assay.

2.3.1 Calculation of standard curves

The means of duplicate readings were then calculated for each standard and sample and the mean zero standard optical density (OD) reading was subtracted. The mean OD absorbance readings for the standard concentrations versus the concentration of the human analyte were plotted and a best-fitting curve was drawn. A seven point standard curve was made using a 2-fold serial dilution in duplicate wells in reagent diluent. A standard curve was run for each group of microwell strips assayed.

The equation used was in the format of $y=mx+c$ with an R value close to 1.000. To determine the concentration of the analyte detected in each sample, the absorbance value on the y-axis was found and a horizontal line was extended to the standard curve. At the point of intersection the vertical line was extended to the x-axis and the corresponding concentration read. The equation was applied to convert each reading and then calculate the means of duplicate or triplicate wells.

2.3.2 Statistical analysis

Secretion levels of the markers assessed were evaluated by a paired Student's t-test with a p value taken at <0.05 to be statistically significant and $p <0.01$ as highly significant. If more than three samples were obtained statistical analysis was performed with inclusion of SD bars.

2.4 Yopro-PI Epithelial cell viability studies

Apoptosis is a highly regulated process of cell death and is distinguished from necrosis by specific morphological and biochemical changes. Apoptosis is followed by activation of $Mg^{2+}Ca^{2+}$ dependent endonuclease and DNA fragmentation as well as cytoplasmic shrinkage and loss of cell membrane asymmetry.

In the early apoptotic state there is a modification of membrane permeability that selectively permits entry of some DNA-binding molecules. (240). YO-PRO is a nuclear dye used to label cells in apoptotic state with compromised cell membranes. It is a fluorochrome that when fluorescing during flow cytometry emits in the green region and carries two positive charges and exhibits high fluorescence enhancement when bound to nucleic acid and does not label living cells (239). YOPRO can enter apoptotic cells whereas other dyes such as propidium iodide (PI) cannot (as shown below in Figure 2.6) (238).

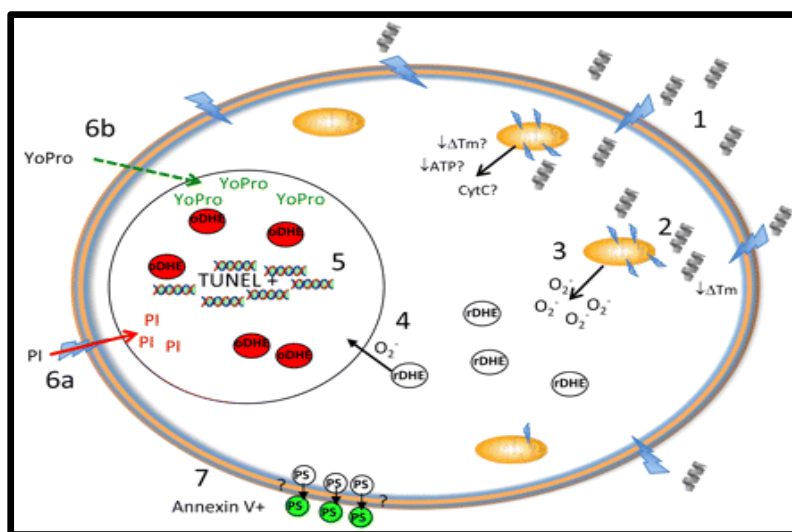


Figure 2.6: Illustration of the YoPro method: PI enters through small pores of oncotic or necrotic cells, resulting in red fluorescing nuclei (6a), whereas YO-PRO-1 enters apoptotic cells that have intact membranes, resulting in green fluorescing nuclei (6b) (238).

Cell death by disruption of cell membranes can be detected by their inability to exclude dyes such as PI. On the other hand cells incubated with sufficient cytotoxic agent to render them incapable of reproducing may exclude PI for hours or even days (239). PI is a commonly used dye to detect dead cells by flow cytometry. PI carries two positive charges which prevents it entering intact cells. When coupled with DNA it is excited by 488nm light to give a bright signal. The binding of PI is reversible and therefore it must be maintained in the extracellular medium during analysis (239). Cells in early apoptosis are unable to pump out YO-PRO but are still not permeable to other dead cells discriminatory dyes. Live cells are not fluorescent or double negative, apoptotic cells are green (YO-PRO +ve) and dead cells are double positive PI+ve /YO-PRO+ve. The cell population should separate in three groups: live cells (green fluorescence), apoptotic cells (increasingly higher level of green fluorescence) and dead cells showing both red and green.

Suspended cells are surrounded by a narrow fluid stream and pass one at a time through a focused laser beam. The light is either scattered or absorbed when it strikes a cell. Absorbed light of the appropriate wavelength may be re-emitted as fluorescence if the cell contains a naturally fluorescent substance or one or more fluorochrome-labelled antibodies are attached to surface or internal cell structures. Light scatter is dependent on the internal structure of the cell and its size and shape. Fluorescent substances absorb light of an appropriate wavelength and reemit light of a different wavelength. Light and/or fluorescence scatter signals are detected by a series of photodiodes and amplified. Optical filters are essential to block unwanted light and permit light of the desired wavelength to reach the photodetector.

The resulting electrical pulses are digitalised and the data stored analysed, and displayed through a computer system. The end result is quantitative information about every cell analysed. Since large numbers of cells are analysed in a short period of time (>1000/sec), statistically valid information about cell populations is quickly obtained. The cells scatter a fraction of the light which is detected by photomultiplier light detectors. A measurement for the diffraction of light horizontally is the forward scatter (FCS) and it depends on the cell volume. A measurement of the diffraction of light in a right angle is the sideways scatter (SSC) which depends on the granularity, the size of the cell, the structure of the nucleus, and the amount of the cytoplasmic contents. The characterisation of the unstained cells using light

scatter is represented using appropriate software in a graph form (Dot Plot) (241,242).

2.4.1 Apoptosis Assay

The experimental protocol for apoptosis assay was followed. Harvested epithelial cells from confluent monolayers were enzymatically dissociated, pooled with non-adherent cells in the culture supernatants, pelleted and washed in cold PBS and cell density adjusted to 1×10^6 cells/mL. For each assay 1 mL assay volume was used. Re-suspended single cells were stained with 0.5 μ M YOPRO-1 (by Molecular Probes) and 4 μ g/mL propidium iodide (by Sigma- Aldrich) were added to the cells and incubated on ice for 20-30 minutes.

After incubation the stained cells were washed in PBS prior to being acquired for flow cytometry (FacsCalibur by Becton-Dickinson, B-D) using 488nm excitation with green fluorescence emission for YO-PRO (530/30 bandpass) and red fluorescence emission for PI (610/30 bandpass). At least 10,000 events were acquired per sample and all flow cytometric data was analysed using CellQuest (B-D). Regions indicating the live cell population were drawn and the mean fluorescence intensity or percentage expression was measured.

CHAPTER 3

RESULTS: IMMUNOHISTOCHEMISTRY STUDIES

Conjunctival epithelial remodelling in CAED and OCP: Expression of epithelial-mesenchymal communication associated molecules.

3.1. Introduction

To determine whether there is epithelial cell remodelling during CAED, a range of molecules associated with remodelling were investigated for their expression in conjunctival biopsies obtained from donors, comparing controls, CAED and OCP. The EMTU related remodelling epithelial molecules selected were EGFR, TGF α and CD44. In the first part of this chapter the antibody concentrations were optimized on a select few biopsies and then, in the second part, the optimal concentrations for each were applied for staining the remaining biopsies.

3.2 Preliminary antibody optimisation immunohistochemistry results

In a CAED biopsy anti-TGF α mAb (1:200 dilution) produced a mild staining pattern of the epithelium with patchy non-specific uptake in the stroma. On the contrary a higher concentration (1:50) produced too intense staining that was not possible to use for evaluating the CK18 expression. The dilution that produced acceptable staining of the expression yet allowing the assessment of the CK18 in the same slide was 1:100. Additionally in a GPC biopsy, anti-TGF α mAb (1:100) produced uniform and milder staining of the epithelium. The grading of the immunostaining with various antibody dilutions is shown in the Tables 3.1 and 3.2 for CK18, EGFR, TGF α , CD44, and IgG.

In a CAED biopsy anti-EGFR mAb (1:200) showed significant uniform expression in the epithelium and diffuse non-specific uptake in the stroma and allowed for assessment of the CK18 expression. However in another biopsy of the same patient, using anti-EGFR mAb at a higher concentration (1:50) produced a too intense staining, whilst anti-EGFR mAb at 1:100 dilution showed brighter and more uniform intensity of staining in the same CAED patient biopsy. In a biopsy of GPC there was no EGFR expression detected for 1:100 dilution, yet the same dilution in a CAED biopsy showed mild positive epithelial detection. The detection of antibodies in the CAED biopsies showed 0 expression for the IgG. Indicative photomicrographs showing CD44 expression at various dilutions in CAED conjunctival sections are shown in Figures 3.1 (A-C).

The remodelling epithelial markers showed co-expression in the epithelium with CK18 and minimal CK18 was detected in the stroma where epithelial cells are absent as expected. Furthermore random CAED biopsies processed for double (3-hour) antibody incubation (as above) showed 0 antibody expression for all antibodies. Based on the data above the final antibody dilutions were determined to be most suitable for the conjunctival biopsies of our Local Tissue Bank.

CK18 epithelial expression		
Antibody dilution	Fields scored x4	Overall score
1:200	1+, 0, 1+, 1+	1+ (uniform)
1:100	2+, 1+, 2+, 2+	2+
1:50	3+, 3+, 3+, 3+	3+ (uniform)

EGFR epithelial expression		
Antibody dilution	Fields scored x4	Overall score
1:200	3+, 2+, 2+, 2+	2+ (diffuse uptake)
1:100	3+, 3+, 2+, 2+	>2+ (patchy)
1:50	>3+, 3+, 3+, 3+	>3+ (very intense)

TGFα epithelial expression		
Antibody dilution	Fields scored x4	Overall Score
1:200	1+, 0, 1+, 1+	1+ (patchy, pale)
1:100	3+, 3+, 3+, 3+	3+
1:50	Non specific	>3+ (too intense)

CD44 epithelial expression		
Antibody dilution	Fields scored x4	Overall score
1:200	3+, 2+, 2+, 2+	>2+ (uniform)
1:100	3+, 3+, 3+, 3+	3+
1:50	>3+, 3+, 3+, 3+	>3+ (very intense)

IgG epithelial expression	
1:200	0
1:100	0

Table 3.1: Preliminary optimization antibody data showing the epithelial grading for various antibody dilutions of randomly selected conjunctival biopsies

CK18 stromal expression		
Antibody dilution	Fields scored x4	Overall score
1:200	0,0,0,1+	0
1:100	1+,0,0,0	0
1:50	0,0,0,0	0

EGFR stromal expression		
Antibody dilution	Fields scored x4	Overall score
1:200	1+,1+,1+,1+	1+ (diffuse, uniform)
1:100	3+,2+,2+,2+	2+
1:50	3+,3+,3+,3+	>3+ (diffuse ,uniform)

TGFα stromal expression		
Antibody dilution	Fields scored x4	Overall Score
1:200	0,0,0,1+	0
1:100	1+,1+,2+,2+	<2+ uniform
1:50	Non specific	>3+ (too intense)

CD44 stromal expression		
Antibody dilution	Fields scored x4	Overall score
1:200	1+,1+,1+,1+	1+
1:100	2+2+3+,3+	>2+
1:50	3+,3+,3+,3+	>3+ (too intense)

IgG stromal expression	
1:200	0
1:100	0

Table 3.2: Optimization antibody data showing the stromal grading scores for various antibody dilutions of randomly selected biopsies.

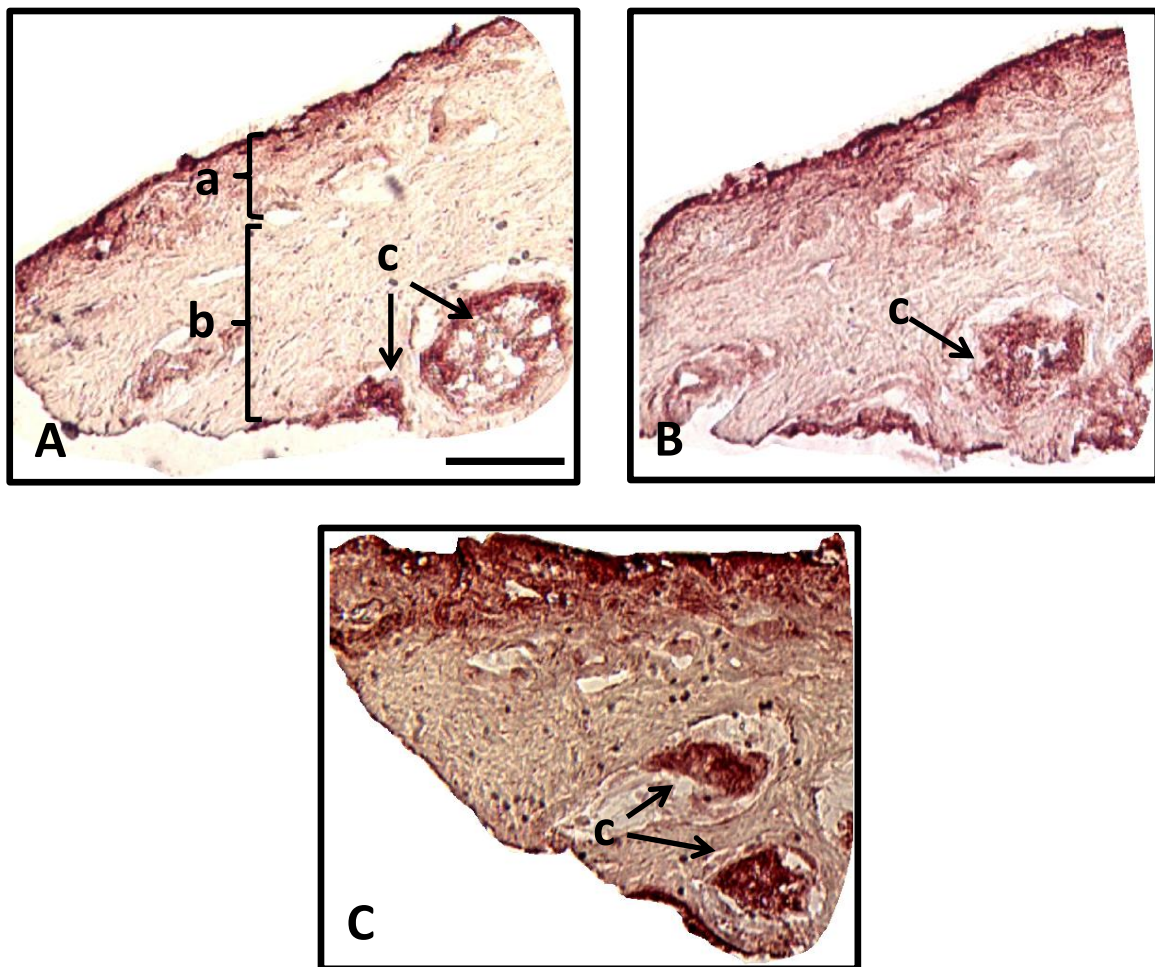


Figure 3.1 (A-C): Example photomicrographs of conjunctival tissue (CAED) from the same donor biopsy specimen used for the CD44 antibody optimisation study at various dilutions (A) 1:200, (B) 1:100, (C) 1:50. In all sections there is intense epithelial staining and stromal antibody uptake. Anti-CD44 mAb (1:200) produced a better staining pattern than the other dilutions enabling improved epithelial expression assessment; Bar shown 250 μ m.

(a) epithelium, (b) stroma and (c) intrastromal epithelial ingrowths indicating papillae formation in CAED.

3.3 Antibody staining

The staining patterns are presented below for each antibody with descriptive analysis of the results in the remaining sections of the chapter. The photomicrographs show the differences in epithelial and stromal expression for each antibody relative to IgG controls, and various conjunctival sections of ocular allergy. Dot plot graphs were used to compare the antibody expression scores and Mann-Whitney statistical analysis performed.

3.3.1 EGFR Immunostaining (1:200)

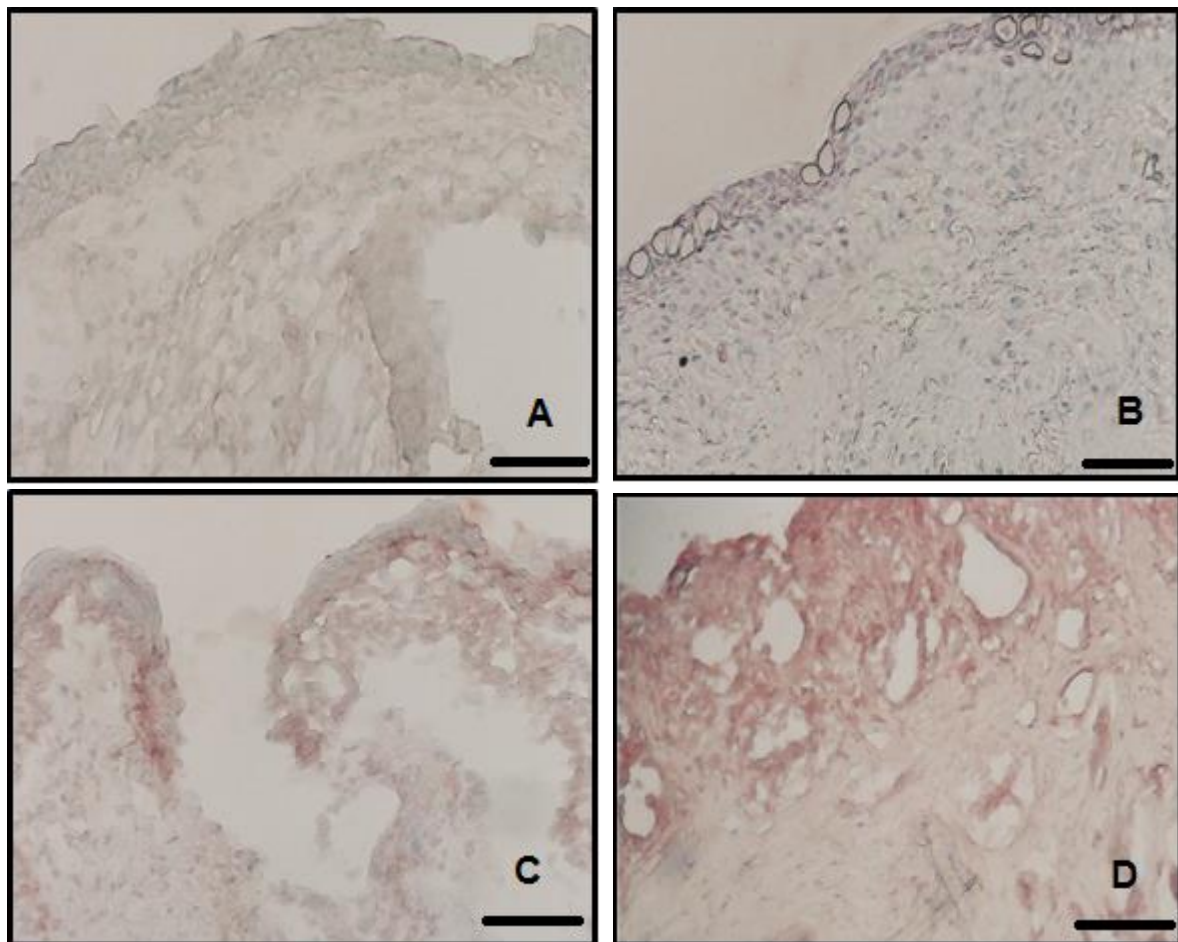


Figure 3.2(A-D): Photomicrographs of sections of conjunctival epithelial demonstrating that the epithelia have different levels of expression for EGFR in normal controls and CAED biopsies. (A) IgG control, (B) No epithelial expression (C) mild (1+) epithelial expression and (D) moderate (2+) epithelial expression with mild stromal staining. Note epithelial cell loss at C and D. Bars shown 50 µm.

3.3.2 TGF α Immunostaining (1:100)

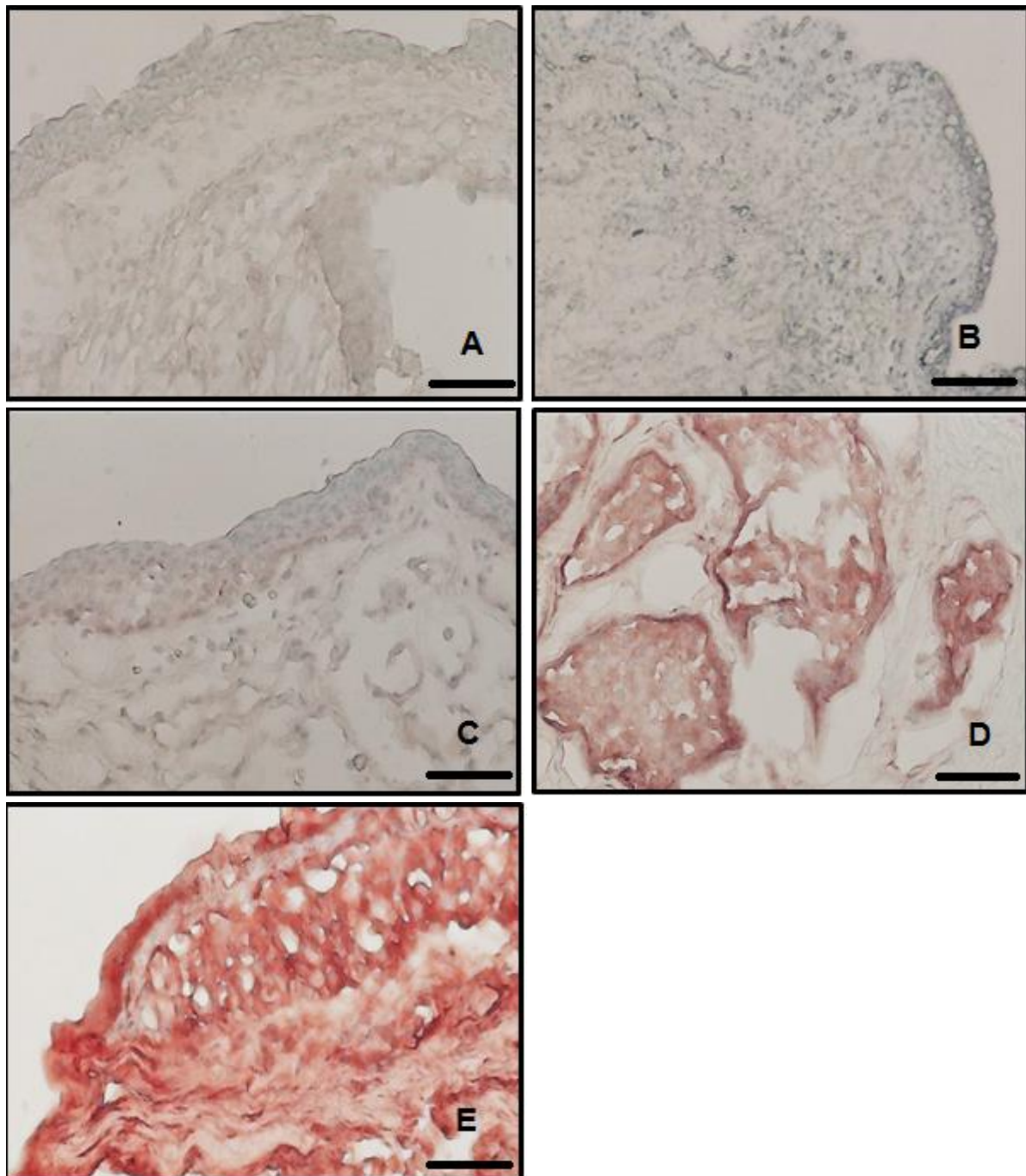


Figure 3.3 (A-E): Photomicrographs of sections of conjunctival tissues showing different levels of TGF α expression in controls and biopsies of CAED. (A) IgG control, (B) 0 expression, (C) (1+) mild expression seen in basal epithelial area, (D) moderate (2+) expression of the intrastromal epithelial areas differentiating them from the surrounding stroma, (E) intense (3+) epithelial expression with stromal staining. Bars shown 50 μ m.

3.3.3 CD44 Immunostaining (1:200)

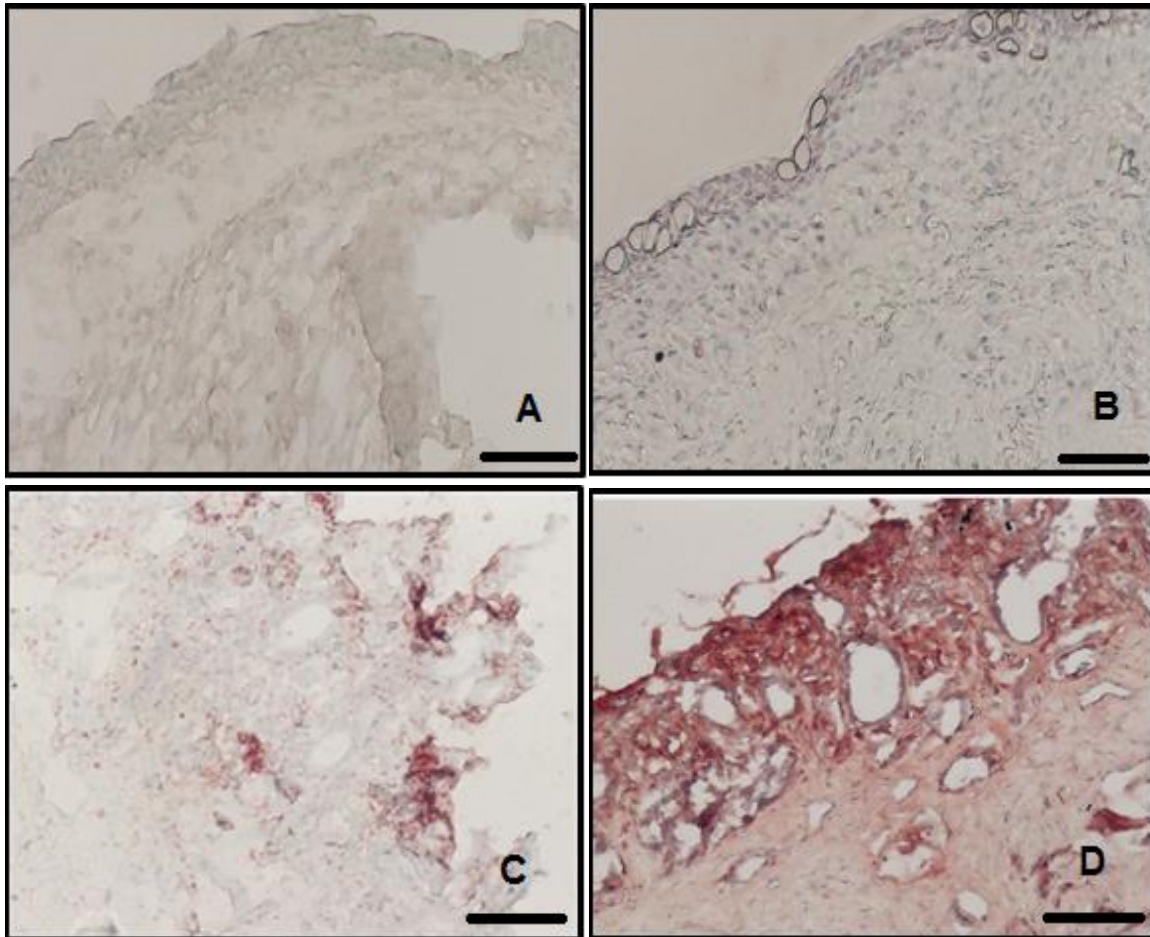


Figure 3.4 (A-D): Photomicrographs of sections of conjunctival epithelia showing different levels of CD44 expression in normal controls and various types allergic conjunctivitis. (A) IgG control, (B) 0 expression, (C) (1+) mild patchy epithelial expression, (D) intense (3+) epithelial CD44 expression and uniform mild (1+) stromal staining. Note epithelial cell loss at photomicrographs C and D (CAED). Bars shown 50µm.

3.3.4 Summary of immunostaining data

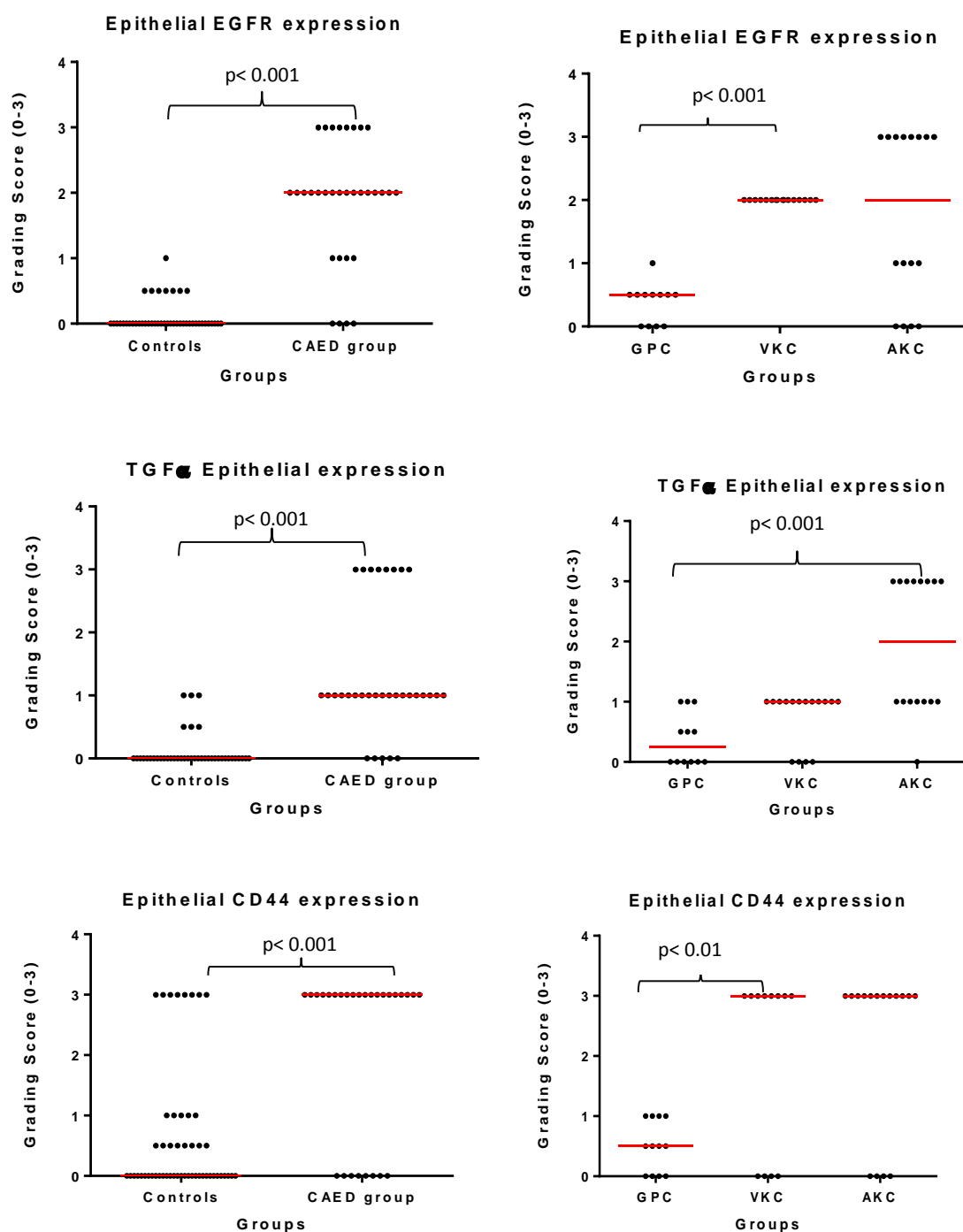


Figure 3.5: Dot plots showing the individual epithelial grading scores for each biopsy of EGFR, TGF α , CD44 expression with median values shown by red lines for each group. p values were calculated using Mann-Whitney analysis.

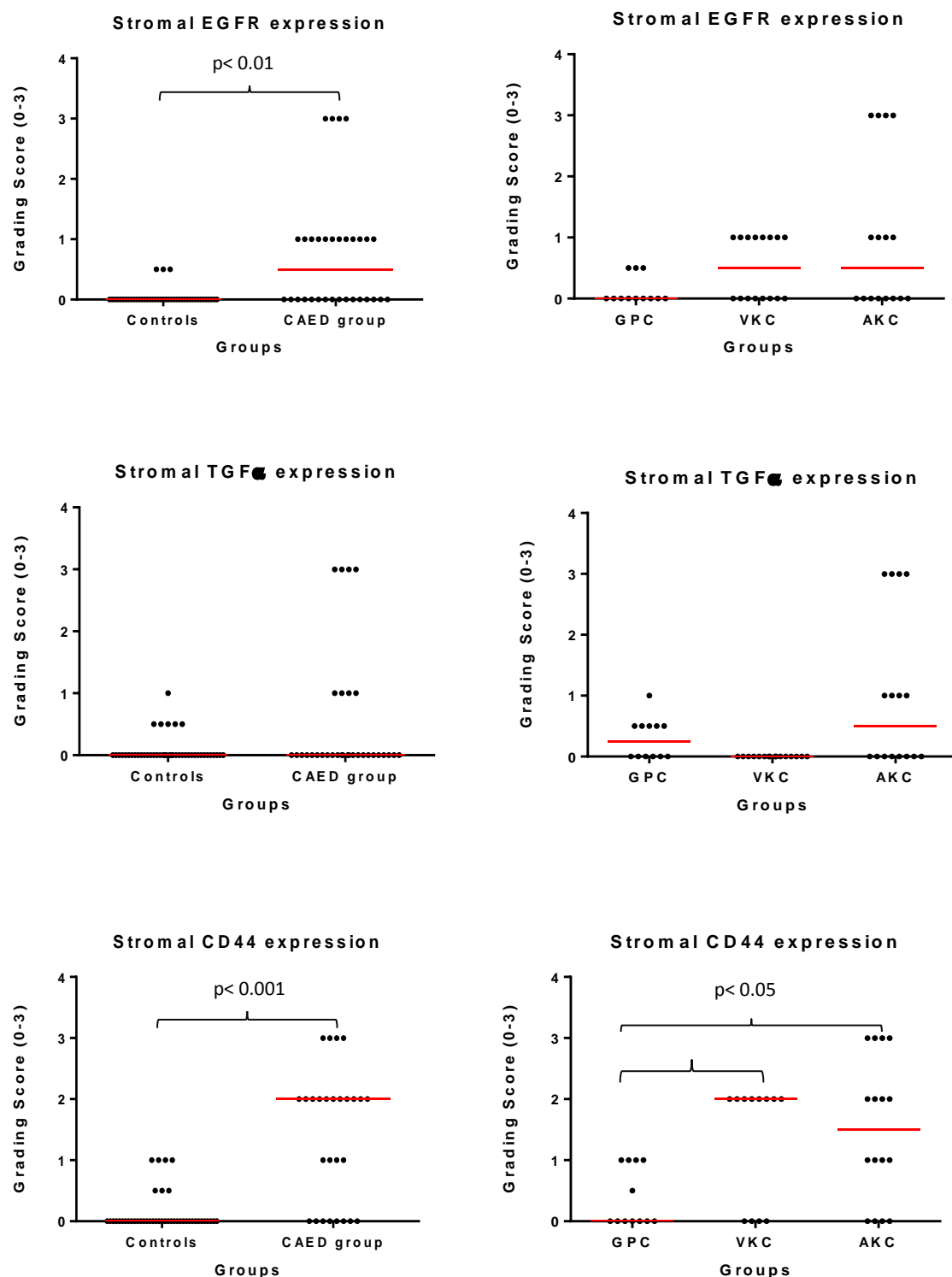


Figure 3.6: Dot plots showing the individual stromal grading scores for each biopsy of EGFR, TGFα, CD44 with median values shown by red lines for each group. p values were calculated using Mann-Whitney analysis.

Stromal antibody expression

Grading Score (0-3)

Antibody

Controls

CAED Group

TGF α EGFR CD44 TGF α EGFR CD44

Antibody	Group	Grading Score
TGF α	Controls	0
		0.5
		0.5
		0.5
		0.5
	CAED Group	1
		3
		3
		3
		3
EGFR	Controls	0
		0.5
		0.5
		0.5
		0.5
	CAED Group	1
		1
		1
		1
		1
CD44	Controls	0
		0.5
		0.5
		0.5
		0.5
	CAED Group	1
		1
		1
		1
		1
TGF α	Controls	0
		0
		0
		0
		0
	CAED Group	1
		1
		1
		1
		1
EGFR	Controls	0
		0
		0
		0
		0
	CAED Group	1
		1
		1
		1
		1
CD44	Controls	0
		0
		0
		0
		0
	CAED Group	1
		1
		1
		1
		1

Figure 3.8: Dot plots summarizing the stromal grading scores of EGFR, TGF α , CD44 with median values shown by red line for each group.

3.3.5 OCP Immunohistochemistry Results

There was no expression of EGFR and TGF α in all normal biopsies similar to that seen in biopsies from normal conjunctiva and seasonal allergic conjunctivitis in the control group in the previous ocular allergy immunohistochemistry study. Additionally both active and chronic OCP groups showed a lack of expression of EGFR and TGF α .

Mild epithelial expression (1+) of CD44 was observed in both groups (chronic and active OCP). In the active OCP group there was positive expression in 7 out of 9 slides expressing the antibody. In the chronic treated OCP group there was positive CD44 expression in the epithelium in 2 out of 6 biopsies. In both groups CD44 expression was uniform and mild, localized in the epithelial layer. There was no expression in the control group. p21 expression was not detected in the chronic OCP group (four biopsies). Table 3.4 below shows the grading results of the OPC biopsies and the normal conjunctival tissues.

Active OCP

Antibody	Epithelial expression	Stromal expression	Number of biopsies
EGFR	0	0	9
TGF α	0	0	9
CD44	1+ in 7/9; 0 in 2/9	0	9

Chronic Treated OCP

Antibody	Epithelial expression	Stromal expression	Number of biopsies
EGFR	0	0	6
TGF α	0	0	5
CD44	1+ in 2/6; 0 in 4/6	0	6
p 21	0	0	4

Normal Conjunctiva

Antibody	Epithelial expression	Stromal expression	Number of biopsies
EGFR	0	0	7
TGF α	0	0	7
CD44	0	0	7

Table 3.3: Overall mean grading scores of EGFR, TGF α , CD44, p21 expression in OCP biopsies.

3.4 Discussion of results

3.4.1 Expression of epithelial remodelling markers in the controls group

All biopsies of normal conjunctival tissue showed a lack of expression of EGFR and TGF α at the epithelium and the stroma without intracellular or extracellular antibody expression. Two of the four normal biopsies showed mild 1+ expression of CD44 in the epithelium and the stroma. This could be due to the fact that during the process of taking the biopsy there was an induced tissue response to injury resulting in secondary expression. These two biopsies showed no expression at the epithelium and the stroma for TGF α or EGFR and the remaining biopsies had no expression. All the other biopsies had undetectable levels of expression. There was mild (1+) expression in two of the three GPC biopsies in the epithelium and much less in the stroma. One of the GPC biopsies showed no EGFR expression but there was some mild expression of TGF α and CD44. The two biopsies of SAC did not show any positivity of any of the antibodies in the epithelium and the stroma.

Two of the three GPC biopsies showed mild (1+) TGF α expression. One showed no expression of the molecule in the epithelium and there was only scanty positivity in the stroma. On the contrary the same biopsy showed positive detection for EGFR and CD44. Possible explanations could include that the amount of tissue TGF- α was not significant enough to be detected or its production had not occurred at the time of biopsy. There was mild (1+) expression of CD44 in the epithelium in all three GPC biopsies. One slide showed equal expression in both the epithelium and the stroma.

Overall, there was mild cytoplasmic and membranous expression of all molecules included in this study in the epithelium and much less in the stroma. The expression was diffuse, less than 1+ and mainly seen in the GPC biopsies. There was no EGFR or TGF α expression in the normal uninflamed subjects.

3.4.2 Expression of epithelial remodelling markers in VKC and AKC (CAED group)

In VKC there was a moderate (2+) EGFR expression (in all four biopsies) in the epithelium and, in two a mild uptake in the stroma. The increased level of expression of the epithelium was statistically significant compared to controls ($p < 0.01$) but not for the stromal expression. Three out of four biopsies showed a mild (1+) TGF α epithelial expression of the antibody. Although this expression was not strong it was statistical significant compared to controls ($p < 0.001$). There was not stromal expression noted. Two out the three VKC biopsies showed strong CD44 expression in the epithelium. The biopsy that failed to express TGF α also did not express CD44 but did show a moderate expression in the epithelium for EGFR and a mild expression in the stroma. CD44 expression in the epithelium was statistically significant compared to controls. Overall there was positive uniform epithelial expression of all three markers EGFR, TGF α and CD44 which was statistically significant compared to the controls group.

One of the four AKC biopsies had no EGFR expression and this could suggest that there are inconsistencies in the pattern of EGFR expression in CAED. The remaining three were EGFR positive. Two were strongly positive and one biopsy showed a weak expression. Expression was mainly epithelial with some patchy stromal uptake. There was statistically significant ($p < 0.001$) expression compared to the epithelial EGFR expression of the controls group but not statistically significant compared to the stroma. There was no statistical significance of the epithelial and stromal EGFR expression between VKC and AKC.

There was positive TGF α expression in all four AKC biopsies. Two showed (3+) strong expression, one biopsy very mild (mean value 0.75+) and the remaining 1+ expression. There was a statistical significance ($p < 0.001$) in epithelial expression compared to the control group but not for the stroma. There was no statistical significance of epithelial and stromal TGF α detection between VKC and AKC. One biopsy was strongly positive for TGF α but showed no detection for EGFR and CD44. Two biopsies showed similar expression scores for both EGFR and TGF α .

There was strong CD44 cytoplasmic and extracellular expression in three out of the four AKC biopsies throughout all fields graded. One of the biopsies showed no detection; the same biopsy specimen which did not express EGFR, was strongly positive (3+) for TGF α . Similar reasons could be attributed for this as described above. However this expression was not statistically significant compared to controls. The same applies for the stromal expression. Three out of the four biopsies expressed significant levels of CD44 in the stroma.

Overall, TGF α , EGFR and CD44 were strongly and uniformly expressed in the epithelium with some stromal expression in the VKC and AKC biopsies despite a few negative fields scoring for some biopsies and this was statistically significant between the control and the CAED group for all markers.

The precise mechanisms by which remodeling is promoted in chronic allergic conjunctivitis are not clear. With the exception of VKC, little is known about the role of growth factors and remodelling in the pathogenesis of chronic allergic eye disease. This is the first study to investigate and compare, by means of immunohistochemistry, the expression of epithelial remodelling markers (EGFR, its ligand TGF α and CD44), in reversible and irreversible forms of allergic conjunctivitis and attempt to explore the possible application of the EMTU hypothesis in asthma to ocular allergy. It is possible that the intense EGFR, TGF α , CD44 expressions in the conjunctival epithelium in CAED may reflect the repair of damage induced by the chronic inflammation. However, an abnormal overhealing response to attempt repair of the damage may be a potential explanation for conjunctival epithelial remodelling.

Initially, a series of immunohistochemistry studies were undertaken to determine the optimal antibody concentrations of epithelial EMTU-associated remodelling biomarkers: EGFR, its ligand TGF α and CD44 acting as a positive control. A control group of conjunctival biopsies (including normal tissue, SAC, GPC) was compared to a clinically severe disease group (AKC and VKC) and by means of immunohistochemistry the epithelial expression of the markers above was graded and analysed. Furthermore immunohistochemistry studies were performed with conjunctival biopsy specimens from patients with acute or chronic OCP aiming to

assess the expression patterns of EGFR, TGF α and CD44. There was a statistically significant difference in epithelial expression of EGFR, TGF α and CD44 between the controls and CAED groups and all ocular allergy patients had active disease at time of biopsying. The OCP biopsies showed no expression of the TGF α and EGFR and minimal CD44 expression in only two biopsies. This may suggest that the remodelling seen in severe ocular allergic disease with corneal involvement, is driven by the epithelium and is not of “autoimmune” origin as in OCP where the fibroblast is known to play a more central role. The figures below show the comparison of EGFR expression in biopsies from SAC, VKC and OCP.

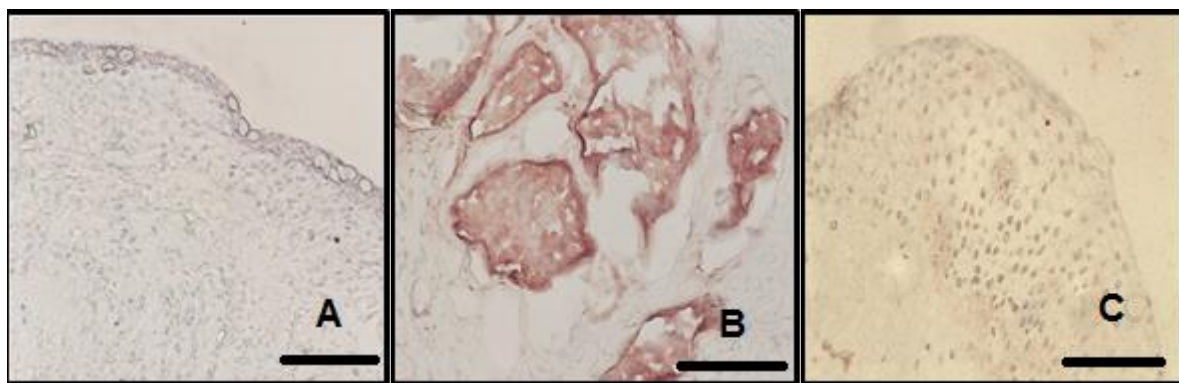


Figure 3.10: Photomicrographs of sections of conjunctival epithelia showing different epithelial EGFR expression in (A) seasonal allergic conjunctivitis, (B) vernal keratoconjunctivitis, (C) ocular cicatricial pemphigoid. Bar shown 50 μ m.

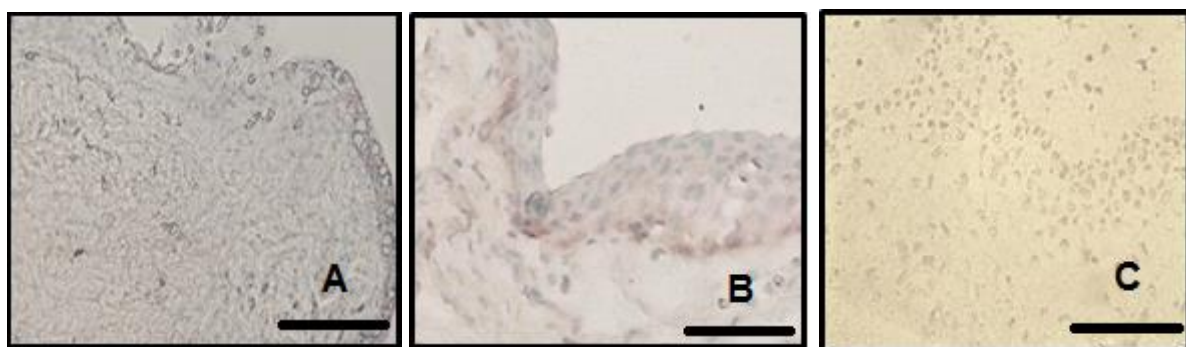


Figure 3.11: Photomicrographs of sections of conjunctival epithelia showing different epithelial TGF α expression in (A) seasonal allergic conjunctivitis, (B) giant papillary conjunctivitis, (C) ocular cicatricial pemphigoid. Bar shown 50 μ m.

Limitations of the optimisation immunohistochemistry studies include the small number of biopsy specimens available for use in this study and the relatively long-term storage at -70°C since they were obtained previously, for studies of our group, which may have influenced the quality of the tissue. GMA embedded conjunctival biopsies with significant tissue destruction were not included. GMA was the preferred technique in this study for fixation since it is known to preserve the tissues for longer and it allows more antibodies to be used. To prevent non-specific staining in positive controls excess washing buffer was carefully removed and freshly prepared reagents were used throughout the process. Incubation times were determined and it was concluded that for the given specimens an overnight single antibody staining was more appropriate to prevent difficulty with the interpretation of the expression of each antibody since in some tissues, strong positivity was anticipated.

The cytokine microenvironment of the inflamed tissue may influence EGFR expression leading to differential and selective upregulation on some cells able to express the receptor. Other reasons that may explain the variable levels of EGFR expression include the degree of clinical severity, the type of inflammation, the cell types involved and the location of the tissue. The expression intensity and pattern may not be consistent across all three areas of the conjunctiva. Variations in the expression patterns could exist between the tarsal, bulbar and limbal areas at different time points of disease progression.

Factors that may influence the variable immunostaining results are presence of a flare-up or a stable chronic not acutely inflamed state and the time of year the biopsy was taken. So far VKC has been the only form of persistent allergic conjunctivitis studied by means of immunohistochemistry to investigate the expression of remodelling markers in the epithelium or stroma. EGFR, VEGF, Collagens I, III, VII and fibronectin in VKC were highly expressed in the sub-epithelial area with collagen I being the major element in VKC compared to controls (123). There is increased diffused epithelial expression of fibrogenic growth factors TGF β , PDGF, FGF in association with increased deposition of collagen in the giant papillae of VKC (127). Geographic variations may also influence the biomarkers' epithelial expression since the disease has been found to be more severe in warmer climates. A lack of EGFR expression has been also reported in VKC where 8 in 16 conjunctival biopsies showed no positive expression of the epithelial receptor (123).

The results of the immunohistochemistry studies described in this thesis add new evidence on the description and comparison of the variable detection patterns of epithelial remodelling markers at different forms of allergic conjunctivitis. The results of this study demonstrate that expression levels of EGFR and TGF α were upregulated in the conjunctiva of patients with corneal involvement and active persistent/chronic allergic ocular disease (AKC/VKC) compared to controls. CD44 not only acted as a positive control but is also known to enhance EGFR signalling and was also found to be overexpressed in the CAED group compared to the controls.

3.5 Summary

EGFR expression was undetected in the epithelium of normal conjunctival biopsies. The overall weak positive expression of EGFR in the controls is accounted for by the mild expression in GPC biopsies. There is no published data to date regarding EGFR, TGF α and CD44 expression in AKC or GPC or the expression of epithelial remodelling markers in reversible compared with chronic forms of ocular allergic disease. The results show that the overall epithelial expression of the markers, EGFR and TGF α , was significantly increased in the CAED group of AKC and VKC in comparison with the controls group. The stromal expression was also increased but mainly in the AKC biopsies with some positivity in the VKC subgroup and it was almost negative for the control group. CD44 and EGFR seem to have the strongest expression in the epithelium followed by TGF α . OCP biopsies showed no expression of the EMTU epithelial markers which supports the suggestion that the EMTU hypothesis may be applied in allergic rather than autoimmune mucosal inflammation involving the conjunctival epithelium.

Although technical limitations may influence the interpretation of the expression of antibody markers in conjunctival biopsies, the results provide immunohistochemical evidence that a similar proposal to EMTU hypothesis may be applicable to AKC and VKC (CAED). However additional evidence is required to reinforce the present data. Immunohistochemistry studies enable the assessment of the detection and pattern of expression of the molecules under investigation and provide a “snap shot” of the tissue at the time when the biopsy was taken. *In vitro* experiments may provide additional data regarding the functions of the epithelial conjunctival cells under inflammatory conditions. Therefore, in the next chapter (Chapter 4) two immortalized human conjunctival epithelial cell lines well known for their properties and used before in ocular allergy research were selected and used as *in vitro* models of human conjunctival epithelial responses: The IOBA-NHC and Wong-Kilbourne derivative of the Chang conjunctiva (CHwk).

CHAPTER 4

RESULTS: *IN VITRO* STUDIES

**Responses to inflammation-related molecules by conjunctival epithelial cells:
An in vitro model of ocular inflammation and abnormal repair.**

4.1 Introduction

This part of the study, aims to develop an *in vitro* model to focus on remodelling markers of epithelial repair and to determine if the EMTU hypothesis can be applied to the conjunctiva, supporting the results shown in Chapter 3. By comparing human conjunctival epithelial cells that were treated with potential stimuli once, it was aimed to produce an acute model of epithelial inflammation. In order to develop a more severe epithelial cell model, cells received multiple treatments over prolonged times.

Human conjunctival cell lines were chosen since it was intended to avoid species differences. The use of animal models was considered but there was no available mouse model of CAED at the time of doing these experiments. There was no access to primary conjunctival epithelial cell lines. Even if such a line was available it would have required repeat biopsies from the same donor in order to carry out this screening of various treatments. Therefore to determine if conjunctival epithelial cells are capable of secreting the remodelling molecules, *in vitro* studies were designed and performed using IOBA-NHC and CHwK human conjunctival epithelial cell lines. A more detailed account of the morphological and functional properties of the selected cell lines is given in sections 4.2 and 4.3. A key aspect of the single and multiple cytokine treatments *in vitro* models was that the culture medium used for the epithelial cell lines was replaced regularly to assure an adequate level of nutrients were present to maintain viability over longer incubation periods. Furthermore, to ensure that the wells did not become overgrown, cells were placed out in each well at lower densities at the beginning of the assays.

In the first portion of this study, preliminary experiments were carried out using the IOBA-NHC line to determine the ability of the cells to secrete a range of molecules associated with remodelling after treatment with various cytokine combinations. Furthermore, a second set of experiments using the IOBA-NHC line were performed in an attempt to assess reproducibility and consistency of the responses using the positive control sCD44. Cell viability studies were also performed to determine whether early apoptosis of the epithelial cells had occurred in response to the treatments.

Finally, three cytokine treatments were selected for the remaining experiments based on their immunological properties and the secretory response profiles obtained from the preliminary experiments. The purpose of this part of the study was to determine the profile of remodelling-associated growth factors and other soluble mediators secreted by conjunctival epithelial cells *in vitro* under the effect of proinflammatory (TNF α /IL-1 β) and Th17-type (IL-17) cytokines to further understand the contribution of conjunctival epithelial cells to ocular surface inflammation and repair. Phorbol 12-myristate 13-acetate (PMA) is well known for exerting a wide range of effects to cells and tissues and is an activator of Protein kinase C and hence NF- κ B. It was therefore selected as a positive control known to induce cellular stress.

During the last few years several developments that enhance our understanding of the physiological and pathological properties of the VEGF family have taken place. VEGF-B, -C, -D members of the VEGF family have also been cloned but it is VEGF-A transcription that is activated in tissue hypoxia, by oncogenes and transmembrane tyrosine kinases such as EGFR. Binding of the ligands to the EGFR leads to DNA reproduction and cell proliferation. The selected ELISA kit assay quantitates the levels of the phosphorylated EGF receptor. When the receptor engages the ligand its degradation is accelerated (approximately 5 hours). Upon binding to the extracellular part of the receptor, internalisation, pH change, enzyme activation, re-organisation of the actin filaments and oncogene protein induction occur. Despite the fact that the precise mechanism of signal transduction is still unclear it is assumed that ligand binding to the receptor initiates transduction via tyrosine autophosphorylation and phosphorylation of interacting proteins.

TGF α is known to have a high affinity for EGFR, acts in both an autocrine and paracrine fashion and is involved in a number of processes including angiogenesis and wound healing. Its precursor has an extracellular, a transmembrane and cytoplasmic domain and shedding of the mature soluble TGF α from its integral domain is highly regulated. EGFR activation, calcium and MMPs are among those factors known to regulate its release. TNF α converting enzyme is known to cleave

mature TGF α from its precursor. The ELISA kit selected to assay TGF α is suitable for cell culture supernatants.

ELISA measures soluble CD44 (sCD44) present in supernatant presumably secreted by the CD44-expressing cells as part of a turnover of the CD44 molecule. It is assumed that what is detected in supernatants in soluble form will positively correlate with secretion levels by the epithelial cells. In this study a sCD44 standard was used since it detects all CD44 isoforms comprising the standard extracellular protein regions thus providing a useful insight into different pathological processes. For the assay of intracellular p21, a sandwich ELISA kit was chosen to measure this analyte within cell lysates. The manufacturer's instructions were followed for the preparation of lysates using the provided lysis buffer and frozen until assayed.

4.2 IOBA-NHC and CHwK cell lines: properties and experimental use

4.2.1 Morphological characterisation

The IOBA-NHC conjunctival cell line is the only non-transfected spontaneously arose from a primary culture of human conjunctival epithelium with continuous proliferation (immortalised), established and characterised at the University Institute of Applied Ophthalmobiology (IOBA), University of Valladolid Spain (235). The cells exhibit typical epithelial morphology with high proliferative activity without contaminating cell types. Care was taken to avoid excessive passaging of cells, and possible change in phenotype. For this, cells were only grown up to passage 20, before new vials were defrosted.

Chromosome analysis confirmed the human origin of the IOBA-NHC lines and there is an altered karyotype with marker chromosomes. Though the ability of genetic variation of a cell line is partially responsible for the establishment of a continuous cell line, not all genetically variable cell populations are immortal. Cells have a polygonal shape with abundant mitotic figures. IOBA-NHC cells have a high proliferative ability determined by quantification of Ki-67 positive cells. Epithelial conjunctival markers such as EGFR were not detected by means of immunofluorescence microscopy but were detected by Western Blot and by flow cytometric analyses.

Several mucin genes were expressed in various passages and cells have the capabilities to produce glycoproteins. The cells are also known to respond to pro-inflammatory stimuli. ICAM-1 was expressed in unstimulated cells and upregulated by IFN γ after 24 hours post stimulation for 72 hours detected by means of flow cytometry. HLA-DR was not detected in unstimulated cells but after IFN- γ treatment there was low but detectable levels. Treatment of cells with TNF α lead to an upregulation of ICAM-1 (but less compared to IFN γ) but did not induce HLA-DR expression.

4.2.2 Cytokine responses

Using the IOBA-NHC cell line the profiles of cytokines secreted in response to exposure to IFN γ (Th1- type), TNF α (proinflammatory), IL-4 or IL-13 (Th2- type) were previously investigated (210). The most effective stimulatory cytokine was TNF α followed by IFN- γ . In contrast IL-4 and IL-13 did not induce significant epithelial cytokine production. Eotaxin-1 production was increased after Th2 type and proinflammatory cytokines treatment. Secretion of IP-10, IL-8 and RANTES were all increased following TNF α treatment. To conclude, under artificial inflammatory conditions the IOBA-NHC line secreted various cytokines which are thought to participate in immunodulation of ocular surface allergic eye diseases (194).

4.2.3 Muscarinic and adrenergic receptors

The presence of muscarinic and α , β - adrenergic receptors in IOBA-NHC cell line was previously investigated (242). The cell line maintained expression of neurotransmitter receptors but with unknown functional state. IFN γ upregulated M₂-muscarinic receptor expression but TNF α did not alter expression levels of any receptors. A combination of cytokines (IFN γ and TNF α) induced increased expression of α_2 adrenoceptors. This evidence makes the line of particular experimental use especially when investigating inflammatory immune ocular surface disease (e.g. dry eye, ocular allergy) since neural alterations are implicated in their immunopathogenesis (242).

4.2.4 CHwK cell lines: Morphological and functional characterisation

The Wong-Kilbourne derivative of Chang cell line (ChWK) is spontaneously immortalised from human conjunctival epithelial cells. The cells have a fibroblastic appearance, do not express cytokeratin 4 (a marker of stratified non-keratinised epithelium), but do express EGF, vimentin and Fas (212). Additionally this cell line is phenotypically similar to primary human conjunctival cells lines, expressing ICAM-1, CD44, CD63, HLA-DR under basal conditions. The ChWK cell line characteristics were compared to primary human conjunctival cell line cells and it was reported that the ChWK line shares morphological characteristics with the mesenchymal cells with a dedifferentiated phenotype (212). CD44, which is known to have anti-apoptotic properties, is similarly expressed in both ChWK cells and primary conjunctival cell line cells. HLA-DR was reported to be upregulated after IFN γ treatment. ICAM-1 was detected at basal conditions and upregulated in a dose-response manner after IFN γ or TNF α treatment but IFN γ was more effective (212).

4.2.5 Immunostimulatory properties of ChWK lines

The immunomodulatory role of human conjunctival cells was previously investigated by means of the *in vitro* model of ChWK line (213). There was an upregulation of HLADR and ICAM-1 after IFN γ treatment. IL-4 treatment did not have any effect on HLA-DR or ICAM-1. The ChWK cell line cells are shown to behave in similar manner in terms of their ability to exhibit a proinflammatory role (213).

4.3 Examples of standard curves of analytes

As described in Chapter 2, standard curves were obtained for every ELISA for each experiment. Example curves are shown below for each protein.

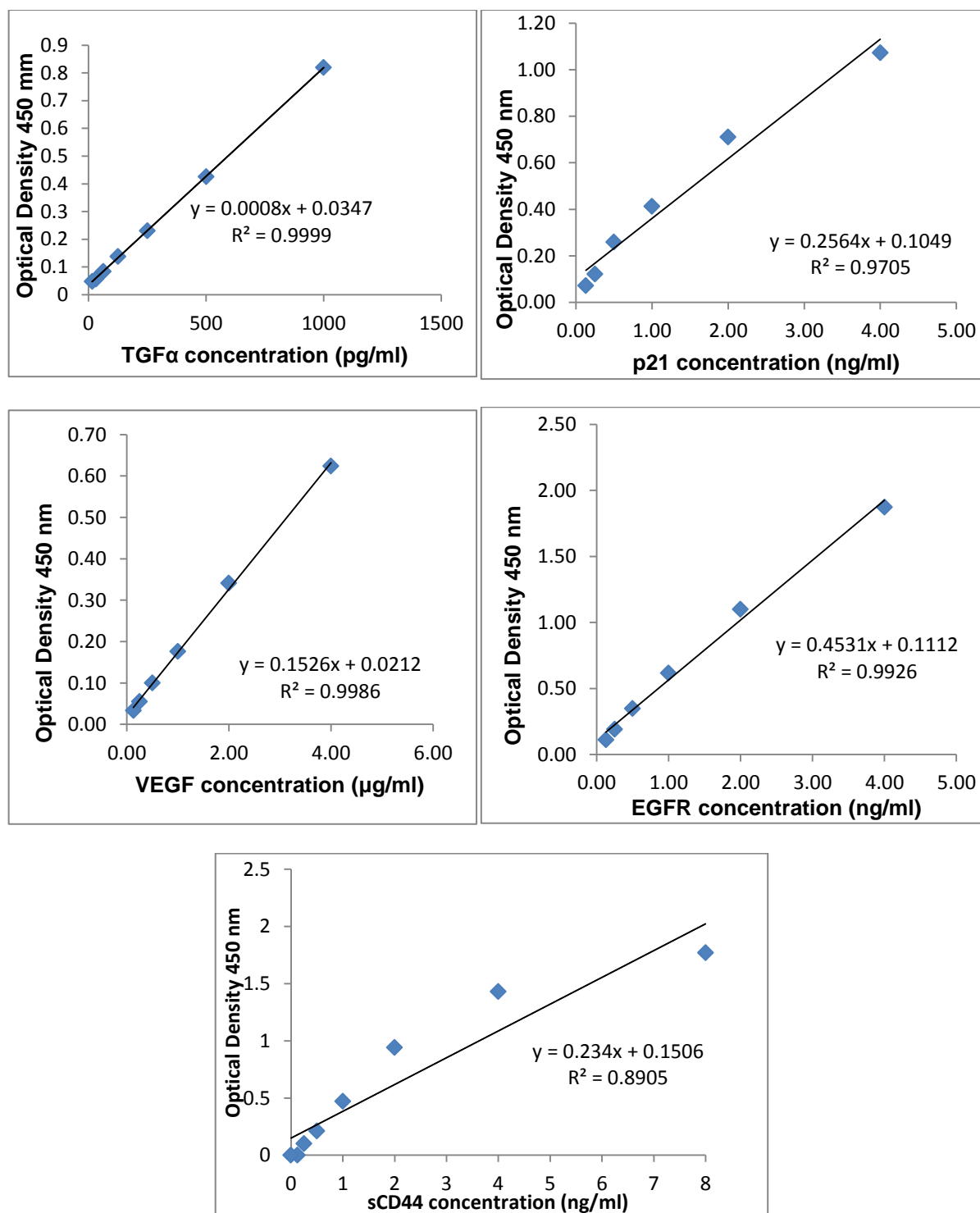


Figure 4.1: Examples of Standard curves produced for all proteins assayed by ELISA.

4.4 Results of *in vitro* pilot study with IOBA-NHC line after a single initial treatment and supernatant harvest at 24, 48, 76, 92 hours

4.4.1. EGFR secretion

In the untreated cells, EGFR was minimally secreted at 24 hours increasing gradually with time, reaching above 0.5 ng/ml at 96 hours. Maximal EGFR secretion occurred in response to exposure to grass pollen [2.0 ng/ml] at 96 hours and was minimal at 24 hours [1.5 ng/ml] level. There was a small gradual increase in its levels for all treatments with increasing time with the lowest levels for the untreated group and maximal for grass. The Figures 4.2 and 4.3 illustrate the EGFR secretion patterns.

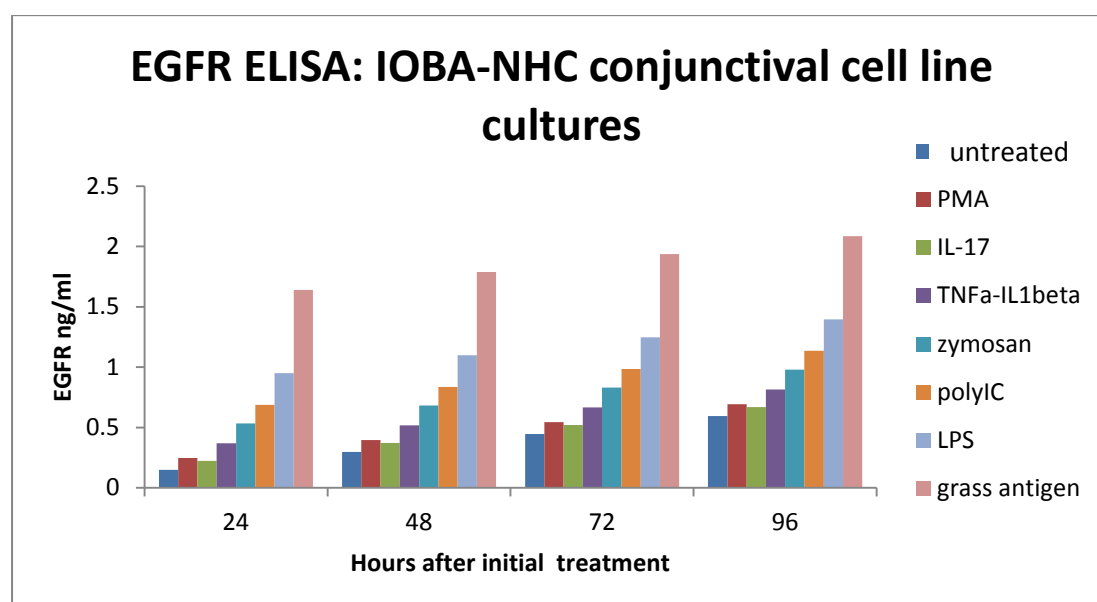


Figure 4.2: Pilot *in vitro* IOBA-NHC study: EGFR expression at 24, 48, 72, 96 hours after various treatment (mean values from duplicate wells)

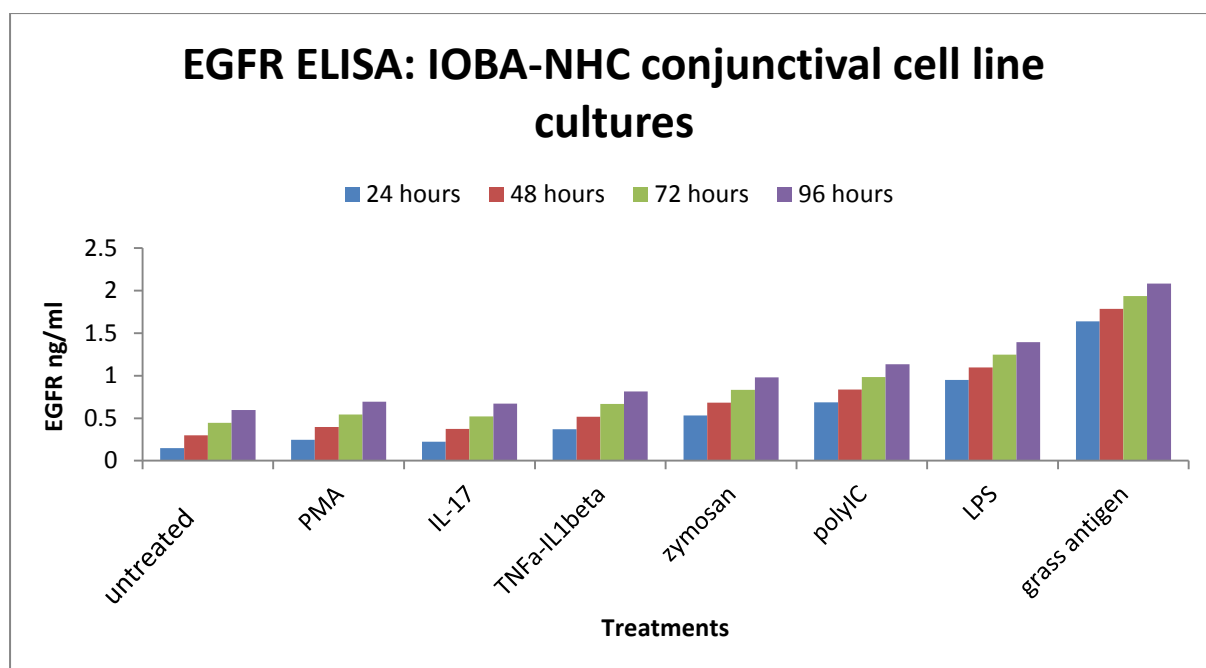


Figure 4.3: Data shown for each treatment indicating maximal EGFR secretion in response to grass antigen. Data is shown as means of duplicate wells.

4.4.2. VEGF secretion

There was minimal VEGF expression at 24 hours for all treatments and an impressive increase at 48 hours which was further increased progressively at 72 and 96 hours. Maximal levels for all groups were detected at 96 hours after treatment. Maximal expression occurred at 96 hours for TNF α /IL-1 β [2450pg/ml] and PMA [2400 pg/ml] as well IL-17A [2100 pg/ml]. The secretion pattern and levels were different than those of EGFR. VEGF levels peaked at later timepoints and demonstrated a greater time-dependent progressive increase than for EGFR. Instead EGFR's levels were more dependent on the type of treatment and had a slower rate of increase in levels. EGFR's secretion appeared to be increasing in a stepwise manner with time for all stimulants. This is not the case of VEGF where there is an abrupt increase at 48 hours increasing until 96 hours for all treatments. Grass pollen appeared to be a more potent inducer of EGFR secretion but not for VEGF production. These variations are shown in Figures 4.4 and 4.5.

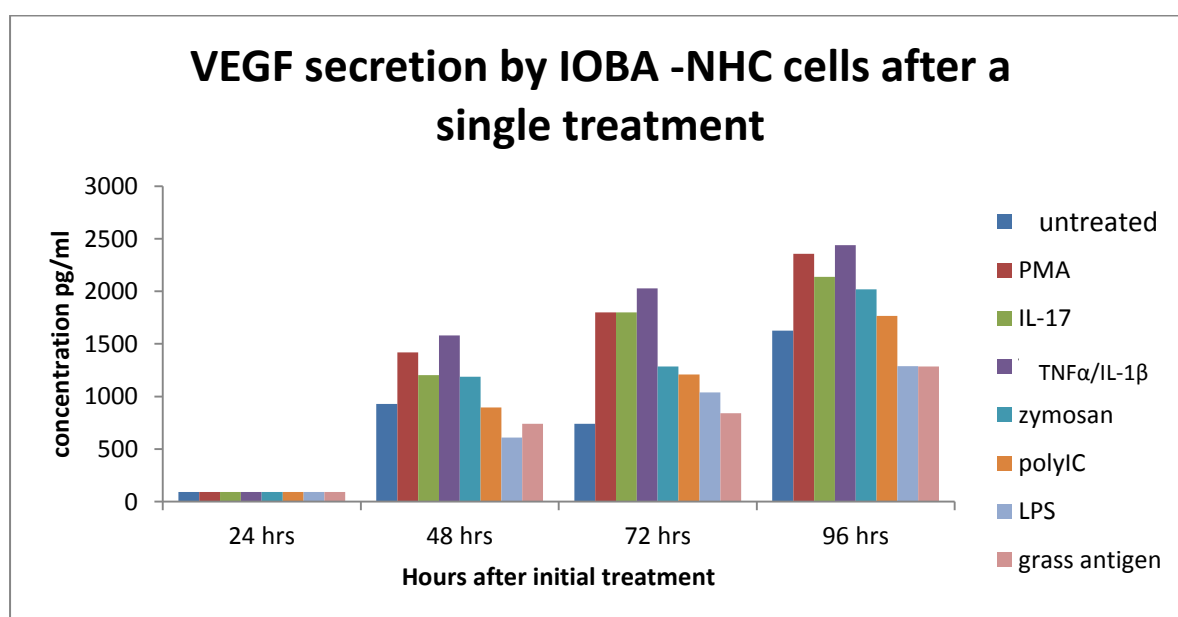


Figure 4.4: Pilot *in vitro* study using IOBA-NHC cells showing VEGF secretion in response to a single exposure with various treatments at 24, 48, 72, 96 hours (based on mean values of duplicate wells).

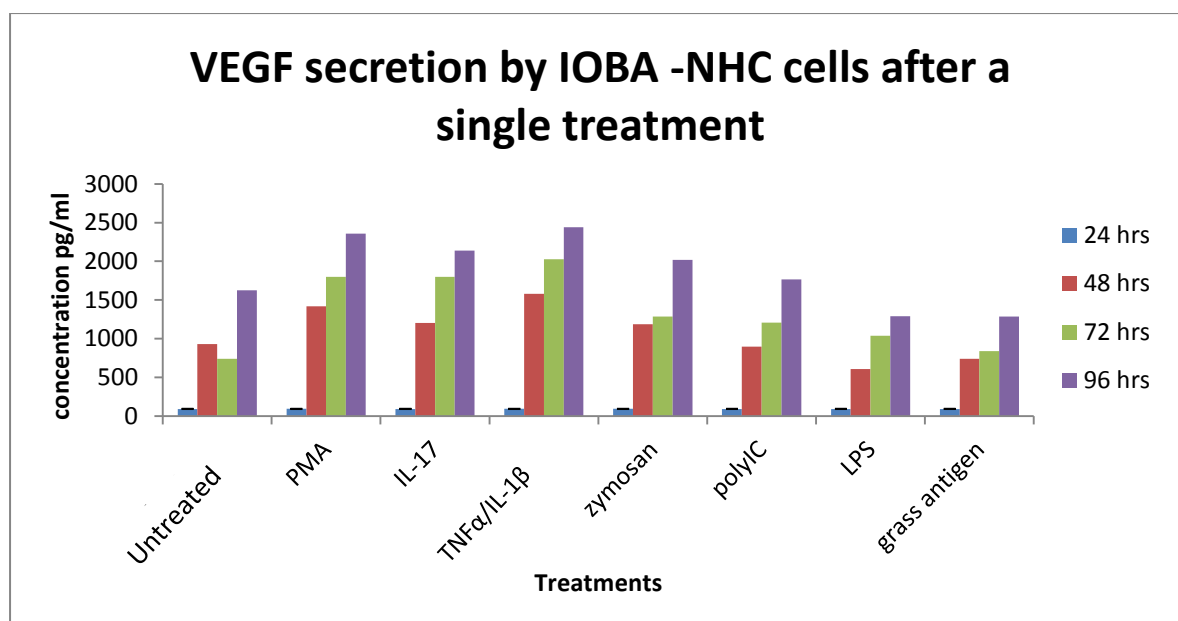


Figure 4.5: Maximal VEGF secretion occurring after a single treatment with TNFα/IL-1β, IL-17 and PMA. Data is shown as means of duplicate wells.

4.4.3. sCD44

There was secretion of sCD44 even in the untreated wells at levels similar to the other treatments at 24 hours suggesting cellular turnover at normal culture conditions. There was no specific pattern of increased detection but the highest levels were found for PMA (7.9ng/ml) at 96 hours. The maximal levels for all groups occurred at 96 hours post-treatment except for the untreated group where maximal secretion occurred at 48 hours. Maximal levels at 96 hours were also detected for IL-17A, TNF α /IL-1 β and grass pollen extract. There was no specific pattern dependent on time or specific stimulants. There was an increase in sCD44 levels at all times for all groups. These changes are shown in Figures 4.6 and 4.7 below.

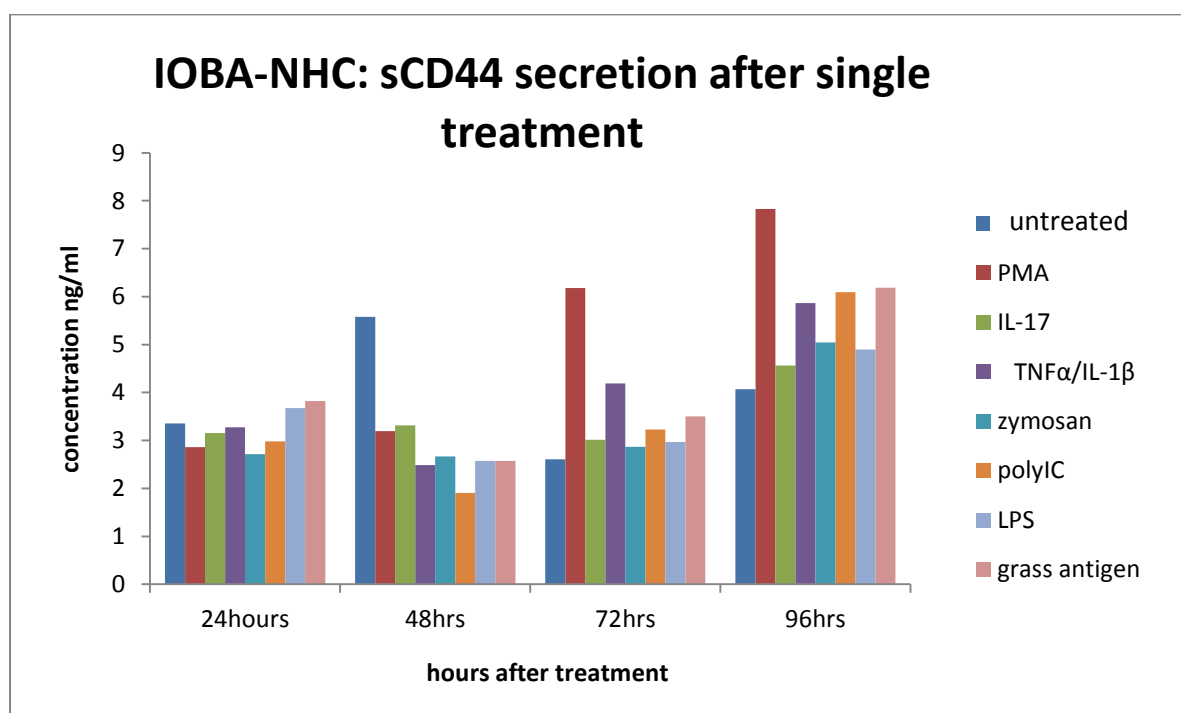


Figure 4.6: Pilot *in vitro* study of IOBA-NHC cells showing levels of sCD44 after treatment with various treatments (based on the mean values of duplicate wells).

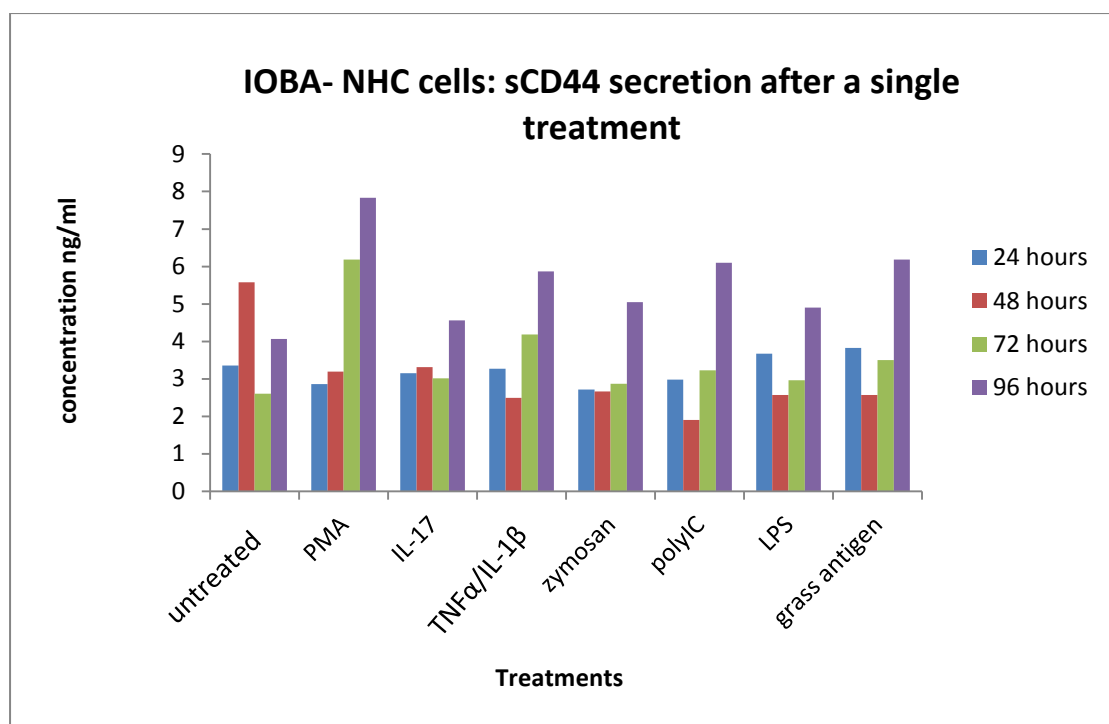


Figure 4.7: Maximal sCD44 secretion occurred at 96 hrs and there was significant turnover of untreated cells even at 24 hours. Data is shown as means of duplicate wells.

4.4.4

TGF α : There was no secretion detected in response to any treatment at any time point. Standard curves were obtained for each assay. This suggested either that secretion was below the level of detection in this ELISA, or that the cells failed to secrete this molecule. Cell death was not observed microscopically. There was no infection noted (data not shown).

4.4.5

p21^{waf}: There was only minimal detection (pg/ml) with large variations for all groups. This minimal positivity could be due to the small cytoplasmic p21^{waf} concentrations (data not shown) and variability could be due to lysate failure. Standard curves were obtained for each assay well.

4.5 Pilot *in vitro* studies: sCD44 secretion by IOBA-NHC cells

4.5.1 sCD44 levels at 24, 48, 72, 96, 144, 192 hours in response to a single cytokine treatment.

In an attempt to determine reproducibility of the sCD44 secretion over longer time periods by IOBA-NHC cells a series of pilot *in vitro* studies using duplicate wells were performed. Production of sCD44 was monitored as a marker of epithelial response regardless the nature of the response profile. The results are shown below in ng/ml. Prior to cytokine exposure the culture medium was replaced to prevent cell loss due to deprivation of nutrients. The secretion levels obtained were overall higher compared to the first study and seemed to follow a similar pattern for each treatment at the various time points. Furthermore maximal secretion was noted at prolonged time points 144 and 192 hours for PMA. IL-17A, PMA and TNF α /IL-1 β induced the highest levels of sCD44. The untreated group also secreted the molecule (Figure 4.8).

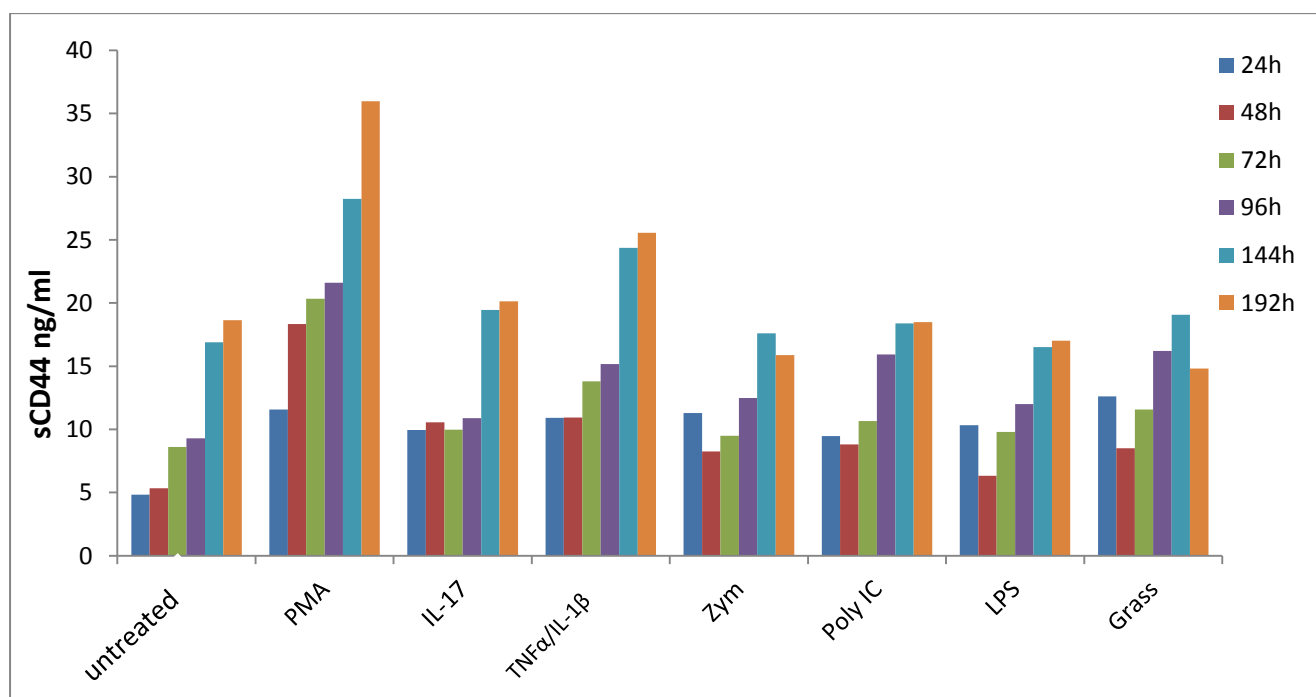


Figure 4.8: sCD44 levels after a single initial treatment by IOBA-NHC cells using various recombinant cytokines at 24, 48, 72, 96, 144, 192 hours based on mean values of two samples.

Furthermore, each treated group was compared to the untreated group and the variability of the production levels of sCD44 is shown (Figures 4.9 and 4.10) during various time points. PMA and TNF α /IL-1 β exposure clearly increased levels of sCD44 at all timepoints. Others have a more variable pattern. The figures for each treatment are shown below. There is a distinct and separate progressive increase in the levels of sCD44 after treatment with PMA or TNF α /IL-1 β compared to the untreated group.

In the untreated group there is also a progressive increase in sCD44 production over time. This reflects cellular turnover increased with time. Zymozan appears to induce similar levels and at some time points equal levels of sCD44, which decrease below the unstimulated levels at 192 hours. Similar patterns are observed by LPS, poly I:C (TLR ligands) and grass pollen extract as shown below.

These cell lines continuously grow and, over longer time periods, could potentially deplete the medium of its nutrients leading to cell death. In these prolonged assays, spent culture medium was replaced with fresh culture medium containing cytokines in each well after each treatment to provide more nutrients and continuous exposure to stimuli in relevant wells. One reason to explain reduction in secretion levels could be overgrowth of cells or cell death. To test this, apoptosis markers were applied and the results are shown in the next section.

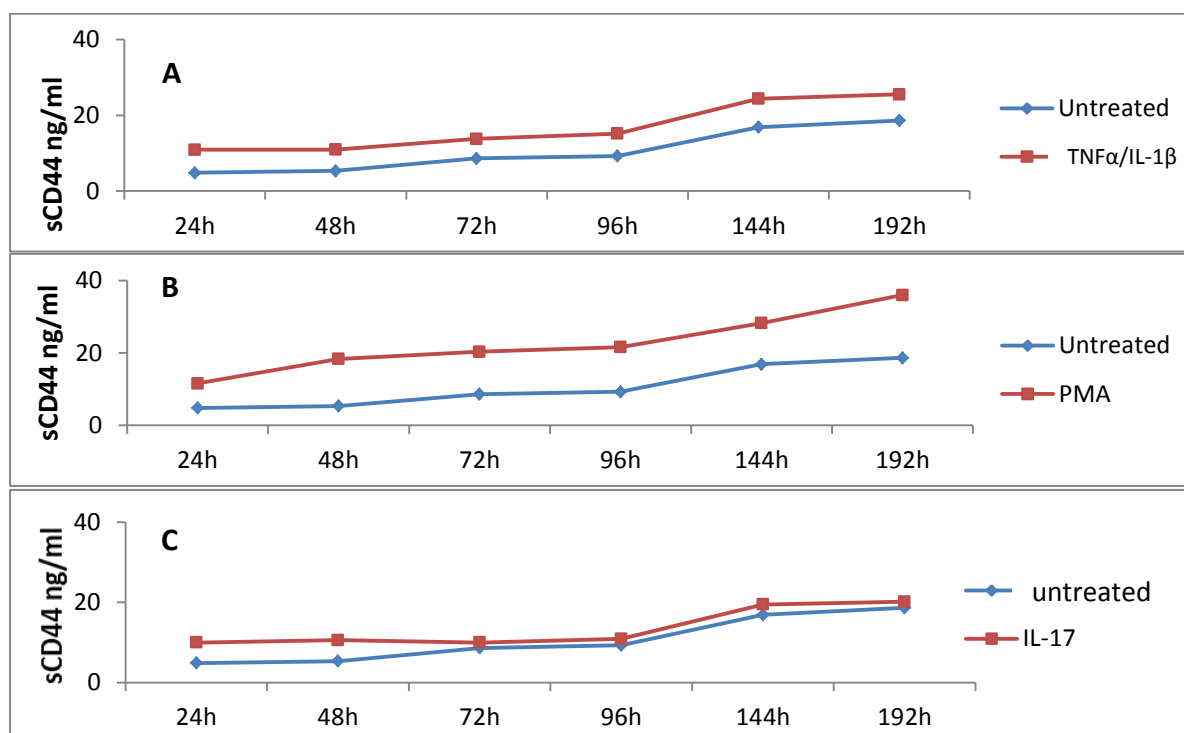


Figure 4.9: (A) sCD44 production after TNF α /IL-1 β treatment compared to untreated cells. The secretion pattern follows that of the untreated cells but at a higher level both reaching a plateau at the final time points. (B) sCD44 production after PMA treatment compared to untreated cells. (C) sCD44 production after IL-17A treatment compared to untreated cells. IL-17A also increased sCD44 levels but was minimally greater than the untreated cells and gradually increased with time.

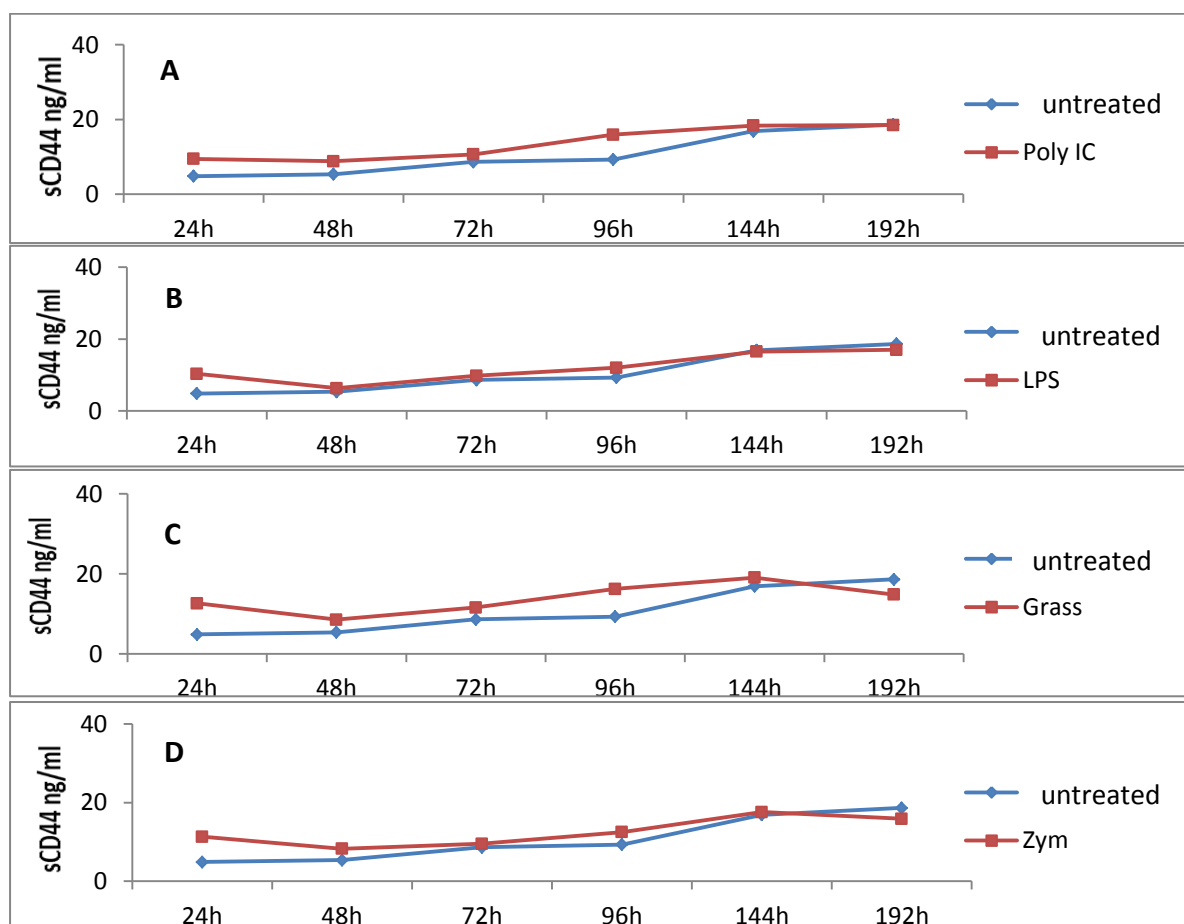


Figure 4.10: (A) sCD44 production after poly I:C (TLR3 ligand) treatment compared to untreated cells. (B) sCD44 production with time after treatment with LPS (TLR4 ligand) compared to untreated cells. (C) Comparison of sCD44 production after grass pollen extract treatment and untreated cells. (D) sCD44 production after zymosan (TLR2 ligand) treatment compared to untreated cells. All data is shown as means of duplicate wells.

4.5.2 Pilot *in vitro* studies to investigate sCD44 secretion at 144 and 192 hours by IOBA-NHC cells after the use of combination of treatments

Further pilot *in vitro* studies were performed to investigate the effect of combination of treatments to the sCD44 production at prolonged time points (144 and 192 hours). It was intended to investigate whether combination of treatments would induce greater secretion levels and supernatants were harvested at prolonged time periods. The results shown below (Figures 4.11 and 4.12) did not reveal significant variations to the previously described results obtained after a single initial treatment. The untreated group showed significant levels of sCD44 indicating cellular turnover at prolonged time points despite the absence of treatments. PMA induced the highest sCD44 secretion levels at both time points and it was maximal at 192 hours. TNF α /IL-1 β treatment also induced significant secretion but less compared to PMA. Prior to cytokine exposure the culture medium was replaced with fresh medium before treating the cells at 24, 48, 72 and 144 hours.

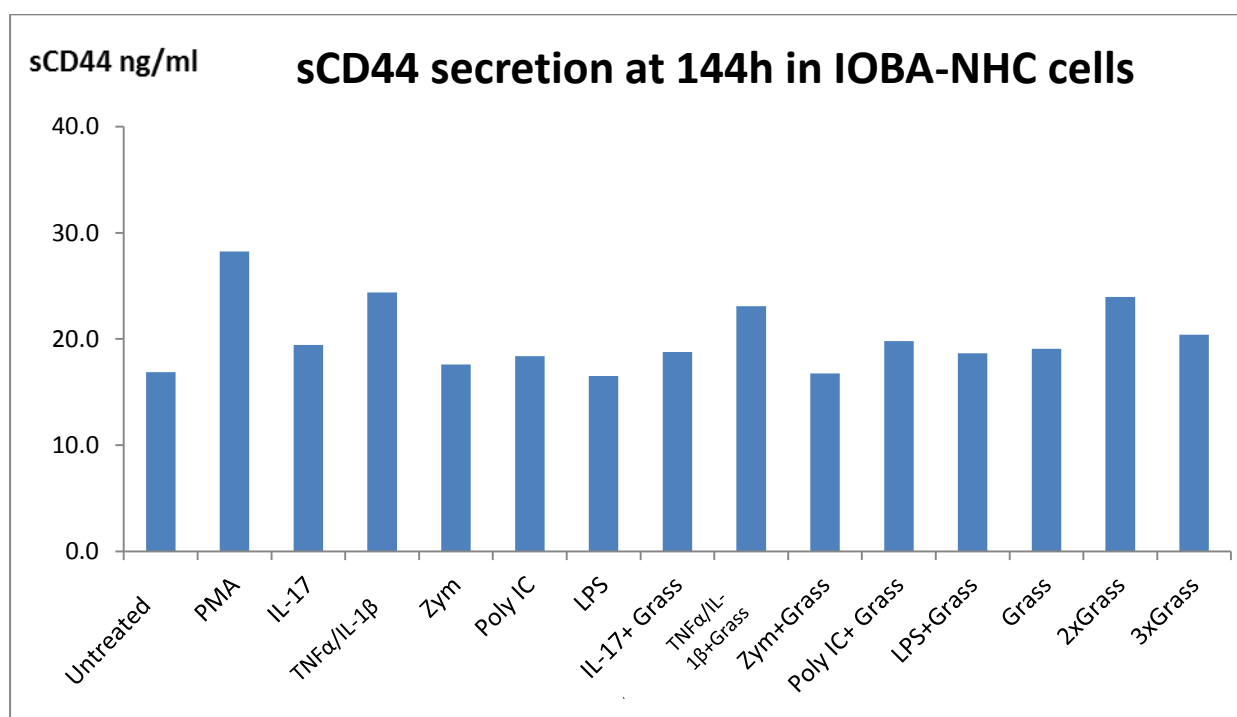


Figure 4.11: sCD44 production by IOBA-NHC cells using various combinations of treatments at 144 hours after treatment (based on mean values of duplicate wells).

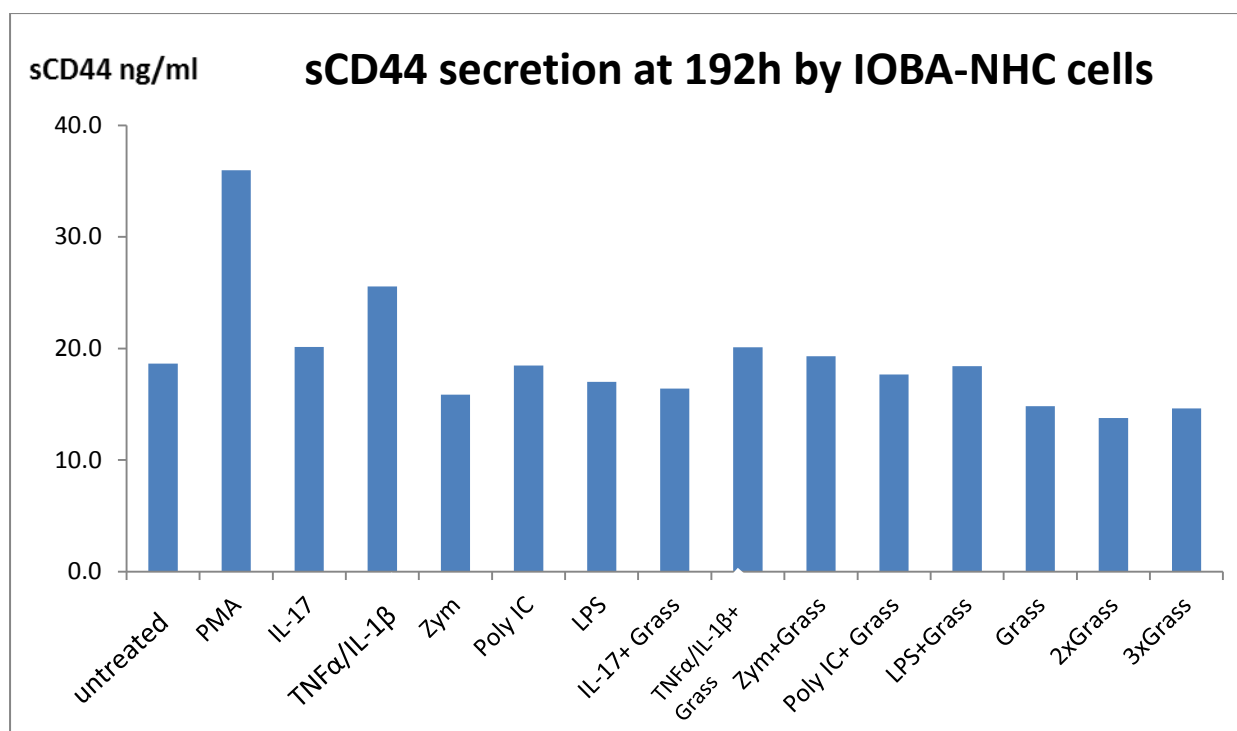


Figure 4.12: sCD44 production by IOBA-NHC cells using various combinations of treatments 192 hours after treatment (based on mean values of duplicate wells).

4.6 Yo-pro epithelial cell viability studies: apoptosis assay results

Epithelial cell viability studies were performed in parallel to the studies described in the previous section. The aim was to assess the viability state of the cell population and determine the proportion of cells that are undergoing apoptosis or are not viable. Samples were obtained from the last set of experiments presented above. Each sample was processed to determine the percentages of the cells in each of the following states: (a) live, (b) dead, (c) in early apoptosis and (d) in late apoptosis. Below the dot plot diagrams show the analysis of results and the percentages of cell populations in each state for the unstimulated group. Similarly the percentages are shown in the remaining diagrams.

Figure 4.13 illustrates the percentages of cell population in the various stages of the cell cycle for the untreated cells. The PMA treated cells were 60% live and 26% in the early apoptotic state. Only 8% were dead. Figures 4.14 show a selection of the cell viability dot plot diagrams. Cells in late apoptosis are of similar percentages (15% and 13% respectively) in both treatment conditions with IL-17A and IL-17A+Grass. Similarly for the cells in early apoptosis (9% and 5% respectively) and cells found dead (35% and 34% respectively).

Furthermore in cells treated with combinations TNF α /IL-1 β and TNF α /IL-1 β +Grass pollen antigen the percentages of the cells in the 4 different stages of the cell cycle appear to be similar: 34% and 30% dead, 13% and 21% in late apoptosis, 5% and 8% in late apoptosis respectively. The results are not indifferent for Zymosan and Zymosan+Grass pollen antigen. This also applies for polyI:C and PolyI:C+Grass pollen antigen, as well as LPS and LPS+ Grass antigen. Additionally, doubling or tripling the Grass pollen concentration did not significantly affect the results.

Therefore in the untreated group, 11% were found in late apoptosis and TNF α /IL-1 β +Grass had the highest percentage (21%). On the contrary PMA-treated cells were found to have the lowest percentages (6%) in late apoptosis and 8% were dead. PMA treated cells showed the highest percentage (26%) of cells in early apoptosis. The addition of grass did not cause a significant change in the percentages of the concentrations. The treatments did not induce early apoptosis at significant levels compared to the untreated group.

Overall, best responses were observed for PMA, TNF α /IL-1 β and IL-17A and combination of treatments with grass extract did not show marked variability. Cell viability studies suggested that the treatments did not induce early apoptosis. Cellular activation was observed even in the untreated group and prolonged harvesting in 144 and 192 hours did not show marked variability on the secretion levels of sCD44. The dot plot diagrams are shown below (Fig 4.14).

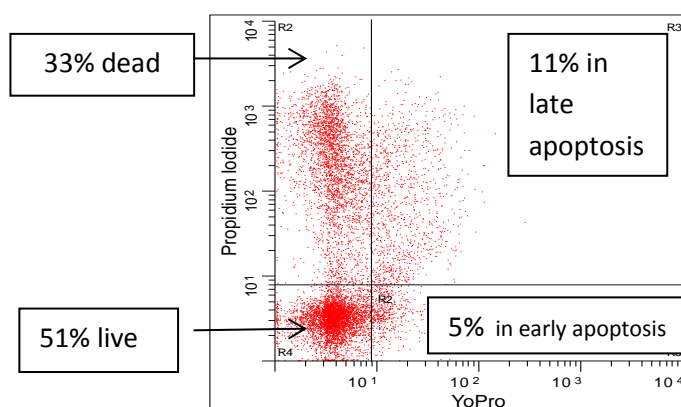


Figure 4.13: Epithelial cell viability assessment for the untreated cells. The annotations show the percentages of the cells in each state of the cell cycle.

Furthermore the remaining experiments were performed and are described in the following sections, where sCD44, VEGF, EGFR, TGF α , p21 secretion levels were assayed by ELISA using two epithelial cell lines.

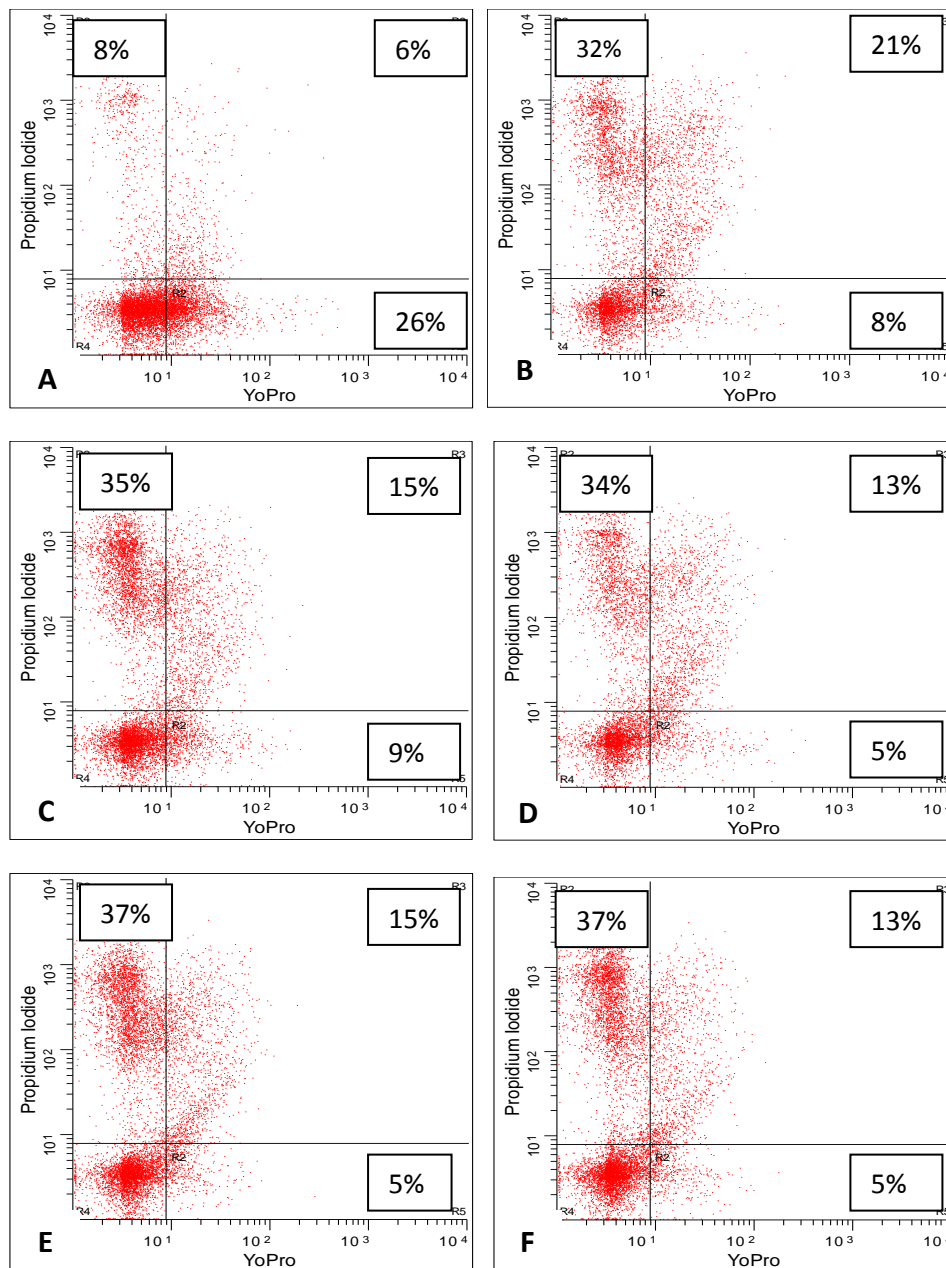


Figure 4.14: Flow cytometric analysis YoPro-PI staining in conjunctival epithelial cells after 192 hours of cytokine stimulation. Examples of dot plots of PI and YoPro were generated from **(A)** PMA treated IOBA-NHC cells, **(B)** TNF α /IL-1 β +Grass extract treated IOBA-NHC cells, **(C)** IL-17A treated IOBA-NHC cells, **(D)** TNF α /IL-1 β treated IOBA-NHC cells, **(E)** Zymozan+Grass treated IOBA-NHC cells, **(F)** Grass extract (triplicated concentration) treated IOBA-NHC cells. The percentages of staining are shown for both markers.

4.7. *In vitro* studies using a single treatment in IOBA-NHC cell lines

Based on the pilot data the experiments were repeated with one initial cytokine treatment (PMA, TNF α /IL-1 β , IL-17A and untreated group) using IOBA-NHC cells and secretion of EGFR, CD44, TGF α was assayed by means of ELISA. Triplicate wells were used for each treatment to allow for a statistical analysis of the responses.

4.7.1. sCD44 secretion

Cells were treated once at time point 0 minutes and supernatants were harvested at 24, 48, 72, 96 hours. sCD44 levels progressively increased with time. This includes the untreated group suggesting cellular turnover. Maximal production occurred after PMA treatment at all time points even in the untreated group (minimal level at 2.5ng/ml in the untreated group and maximal at 96 hours at 13 ng/ml). A similar effect was observed for IL-17A but at much lower levels. TNF α /IL-1 β induced sCD44 secretion peaking at 72 hours just above 6ng/ml and then reducing at 96 hours (6.0 ng/ml). The results are shown in Figure 4.15 below.

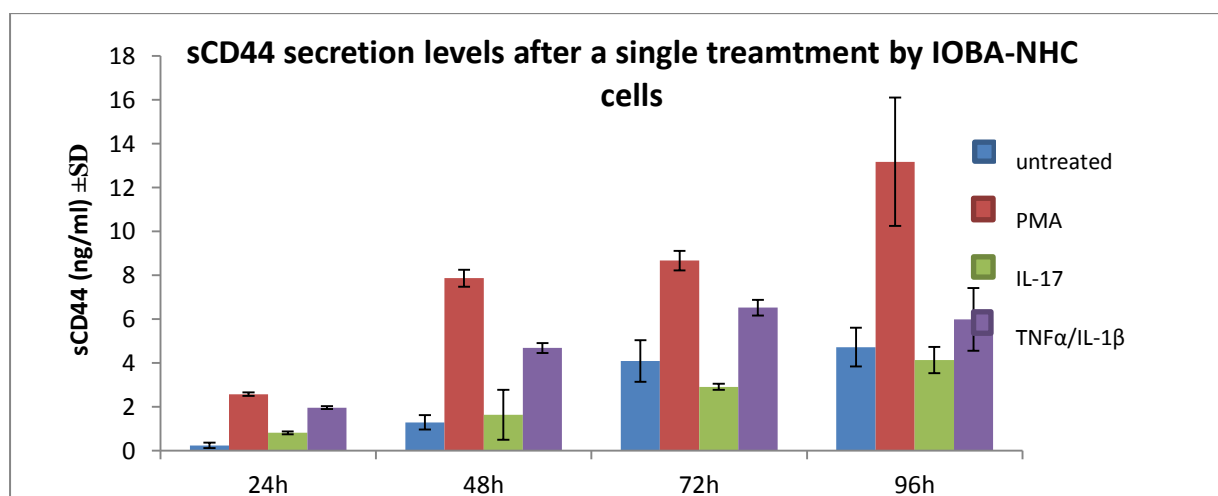


Figure 4.15: sCD44 levels at 24, 48, 72, 96 hours after treatment at time 0 with PMA, TNF α /IL-1 β and IL-17A and untreated group (Data is shown as means \pm SD from triplicate wells).

	t test 24hrs	t test 48 hrs	t test 72 hrs	t test 96 hrs
Untreated vs PMA	p<0.01	p<0.01	p<0.05	p<0.05
Untreated Vs TNF α /IL-1 β	p<0.01	p<0.01	NS	NS

Table 4.1: t-tests shown for sCD44 levels. IL-17A treated groups were not statistically significant to unstimulated - not shown.

4.7.2. EGFR secretion

In the untreated group EGFR secretion was present at 24 hours and slowly increased with time at 96 hours (8ng/ml). It is noteworthy that there was a stepwise slow increase in EGFR secretion in the untreated group. Maximal secretion occurred once again with PMA treatment at 96 hours (>20ng/ml). This pattern is similar to the sCD44 secretion pattern. IL-17A induced EGFR secretion that remained at almost constant levels around 10ng/ml throughout all times. TNF α /IL-1 β treatment also induced EGFR production which increases from 10ng/ml at 24 hours to 14ng/ml at 96 hours. EGFR epithelial secretion increased with time as a response to the cytokine treatments as well as in the untreated group indicating cellular turnover.

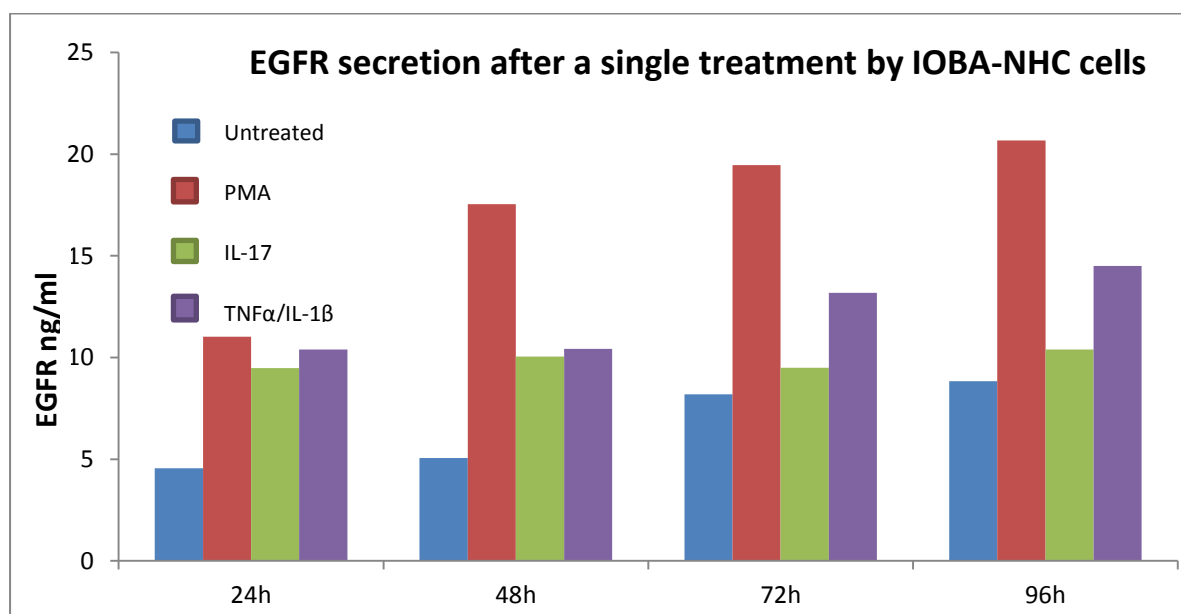


Figure 4.16: EGFR levels at 24, 48, 72, 96 hours after one treatment with PMA, TNF α /IL-1 β , IL-17A and untreated group (based on mean values of duplicate wells).

4.7.3. TGF α secretion

As shown in Figure 4.17, TGF α secretion was detected from 24 hours with a stepwise progression. It declined for all treatment groups at 96 hours. Its maximal secretion occurred at 72 hours after treatment with IL-17A. All groups had maximal TGF α levels at 72 hours. There was no significant variation in the baseline, untreated group, for all time points and there was increased secretion for all groups at most timepoints after treatment as summarised in Table 4.2.

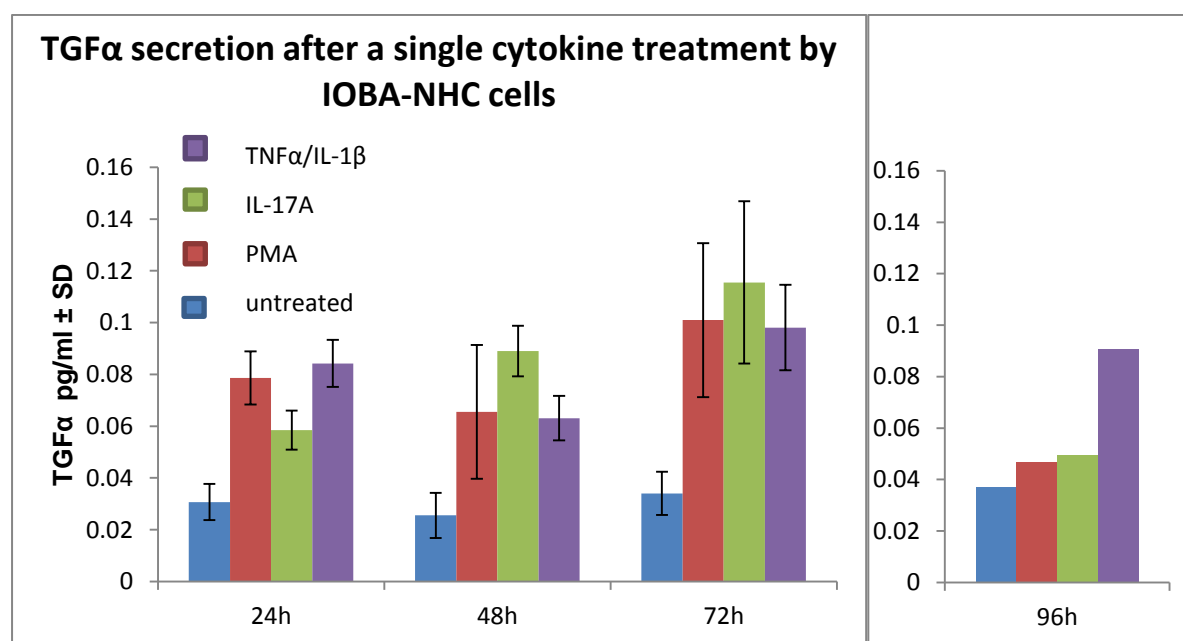


Figure 4.17: TGF α secretion after treatment with PMA, IL-17A, TNF α /IL-1 β at 24, 48, 72 and 96 hours (Data is shown as means \pm SD from triplicate wells except levels at 96 hours for which duplicate samples were used; hence error bars for levels at 96 hours are not included).

t test	24hours	48 hours	72 hours
Untreated vs PMA	p<0.01	NS	NS
Untreated vs TNF α /IL-1 β	p<0.01	p<0.05	p<0.05
Untreated vs IL-17A	p<0.05	p<0.01	NS

Table 4.2: t-test analysis for TGF α secretion levels compared to untreated cells. No statistical analysis was performed for the levels at 96 hours since data were obtained for two samples.

4.7.4 Summary of findings

The following table summarises the responses by the IOBA-NHC cells after a single cytokine treatment. There was increased secretion for all remodelling markers assayed in response to treatment with all cytokines.

Effect on secretion after a single cytokine treatment over 96 hours by IOBA-NHC cells				
cytokine	EGFR	VEGF	sCD44	TGF α
PMA	↑	↑	↑	↑
IL-17A	↑	↑	↑*	↑
TNFα/IL-1β	↑	↑	↑	↑

Table 4.3: Summary of responses of the remodelling markers after a single cytokine treatment. * IL-17A induced increased sCD44 secretion until 48 hours but was found to be reduced at 72 and 96 hours compared to the untreated group.

4.8 *In vitro* studies using multiple treatments in IOBA-NHC cell lines

In this part of the *in vitro* studies, the cells were treated three times with the same treatment and the secretion of EGFR, CD44 and VEGF was assayed by means of ELISA. Supernatants were harvested at 24, 72, 144 and 192 hours and culture medium containing stimuli replaced.

4.8.1. EGFR production

Similarly there was a progressive increase in EGFR secretion with time and repeated treatments. Interestingly, EGFR levels were lower than the ones observed in the once-only treatment studies described in the earlier section. TNF α /IL-1 β induced maximal levels at 192 hours after 3 treatments (0.35 ng/ml). PMA followed and then IL-17A showed a stepwise increase in the EGFR secretion including the untreated cells.

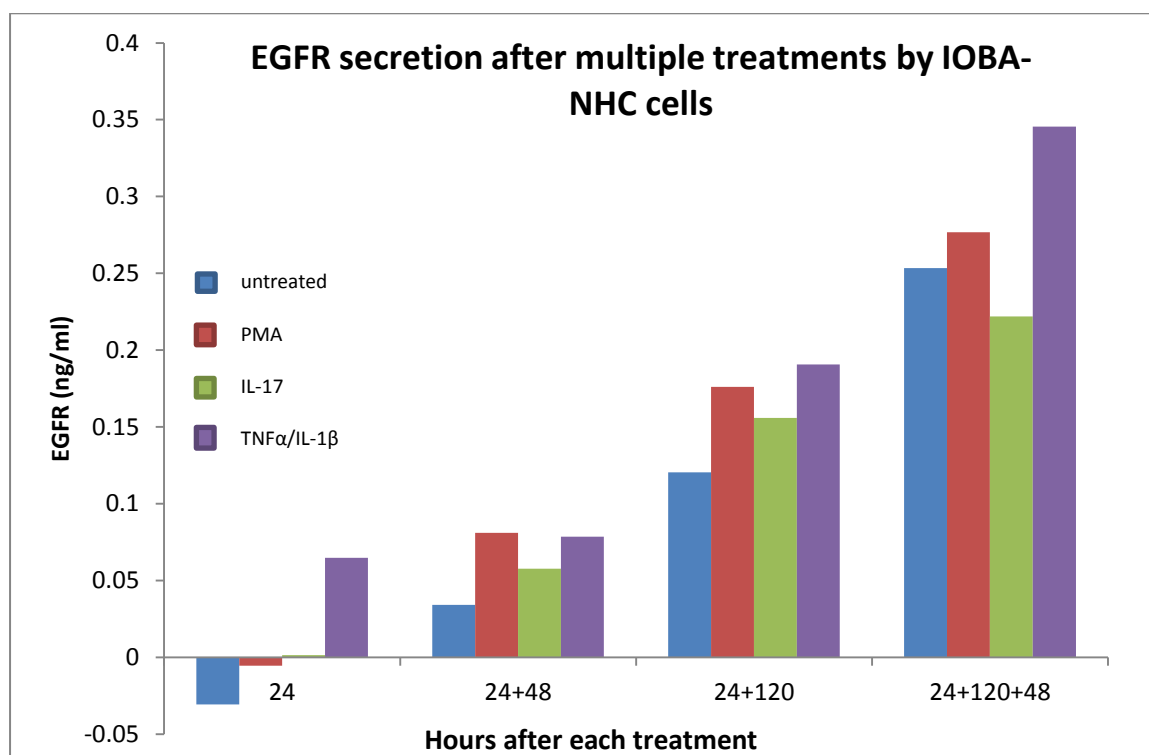


Figure 4.18: EGFR secretion after three treatments at various time points (based on the mean values of duplicate wells).

4.8.2. sCD44 secretion

There was a progressive increase of the sCD44 levels from the first 24 hours for all groups with maximal secretion after two PMA treatments at 144 hours. Furthermore the sCD44 levels decreased at 192 hours after a third treatment as shown in Figure 4.19 below. The reduction in sCD44 levels could be due to toxicity induced by the treatments after prolonged culture periods and therefore a reduction in epithelial cell function. Secondly epithelial repair could have taken place resulting in a reduction of sCD44. EGFR secretion pattern correlates with sCD44 levels except for the final 192 hours sample. There are also similarities with the secretion pattern of VEGF.

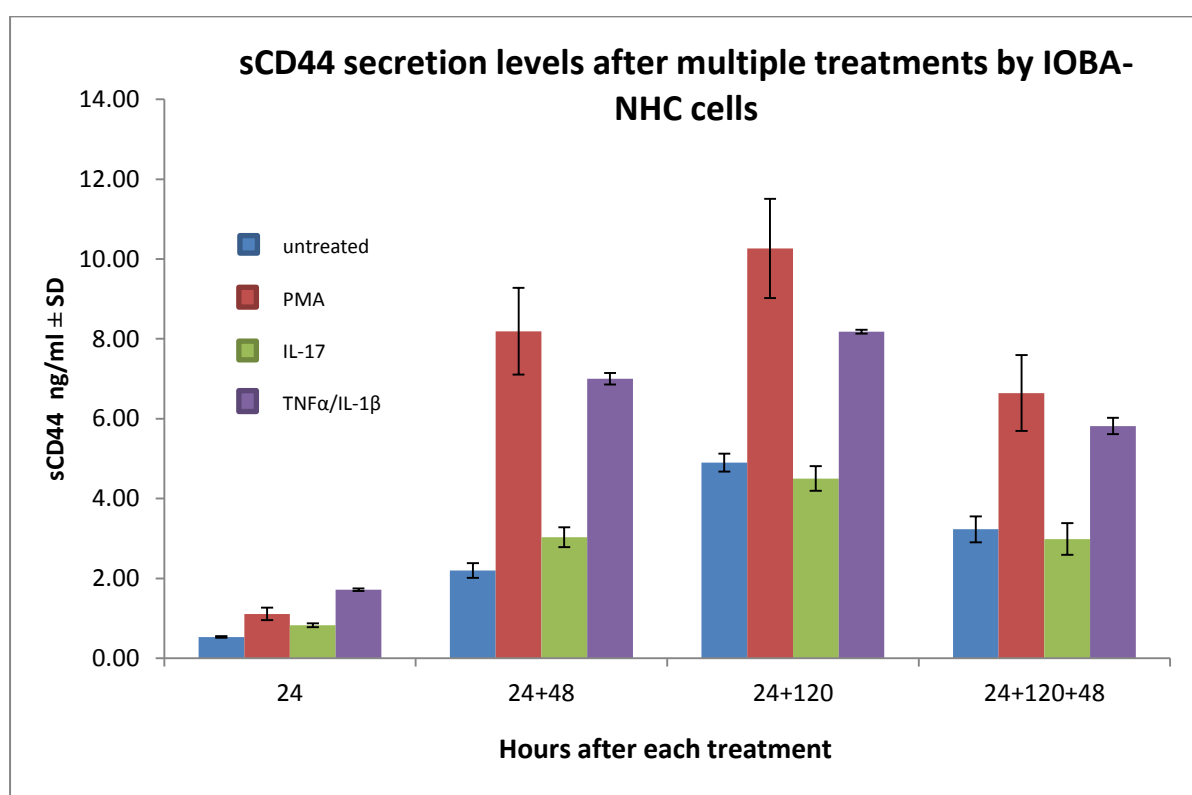


Figure 4.19: sCD44 secretion based on triplicate wells at various timepoints. Data is shown as means \pm SD from triplicate wells.

4.8.3 VEGF secretion

The production pattern is similar to the one observed by the IOBA-NHC line after one treatment in the pilot set of *in vitro* studies. There was a stepwise pattern with minimal levels from the first 24 hours and maximal at 192 hours. Maximal production occurred after three TNF α /IL-1 β treatments at 192 hours [12.0pg/ml]. The stepwise increase applied for all groups with increasing time after treatment including the untreated group. The production pattern of EGFR correlates well with VEGF with progressive stepwise increase with time. Both markers appeared to be present in the first 24 hours but in the pilot *in vitro* study after one treatment EGFR appeared at greater levels earlier than VEGF.

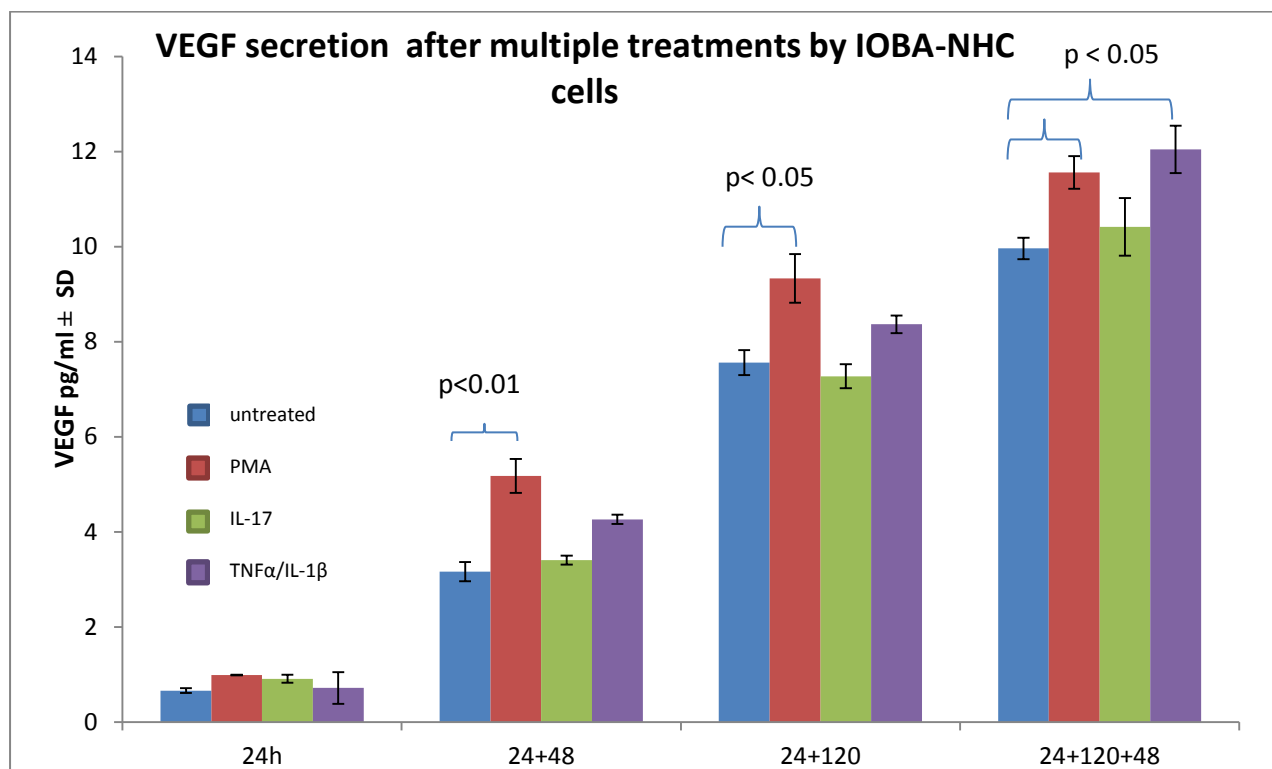


Figure 4.20: VEGF production after three treatments over a period of 192 hours. Data is shown as means \pm SD from triplicate wells.

t test	24 + 48 hrs	24+120hrs	24+120+48
Untreated Vs PMA	p<0.01	p<0.05	p<0.05
Untreated vs TNF α /IL-1 β	NS	NS	p<0.05

Table 4.4: t-tests for VEGF production of untreated vs PMA and TNF α /IL-1 β groups.

4.8.4 Summary of responses

The following table shows a summary of the responses observed by the IOBA-NHC cells after multiple cytokine treatments.

Effect on secretion after multiple cytokine treatments over 192 hours by IOBA-NHC cells			
cytokine	EGFR	VEGF	sCD44
PMA	↑	↑	↑
IL-17A	↑*	↑	↑**
TNFα/IL-1β	↑	↑	↑

Table 4.5: Summary of the secretion of remodelling molecules in response to multiple cytokine treatments by IOBA-NHC cells.

*IL-17 induced increased EGFR secretion compared to the untreated group but at the final time point at 192 hours was shown to be reduced.

**IL-17 induced increased sCD44 levels for the first 72 hours but the levels were reduced compared to the untreated group until the final time point.

4.9 *In vitro* studies using IOBA-NHC cells to investigate p21^{waf} levels after a single treatment with PMA, IL-17A and TNF α /IL-1 β at 144 and 192 hours.

There was increased expression for all treatments above baseline and greatest levels were noted after IL-17A. However the levels produced are at very low concentrations (pg/ml). Levels decreased at 192 hours and were higher at 144 hours after one treatment (0.95 pg/ml with IL-17A treatment and 0.30 pg/ml after TNF α /IL-1 β or PMA. The results are shown below in Figure 4.21. Given the small concentrations of the duplicate samples, statistical analysis could not be performed.

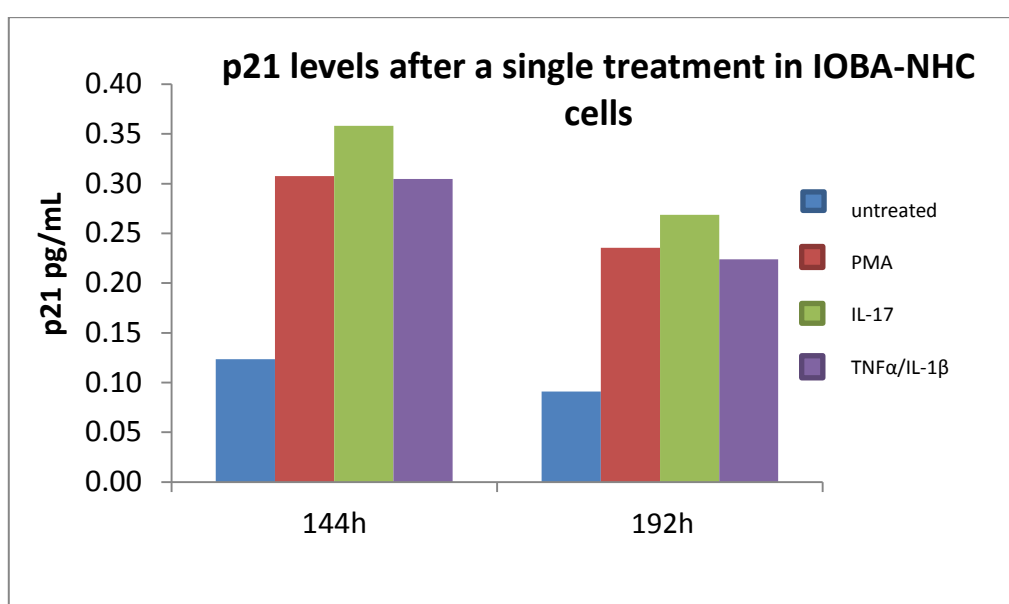


Figure 4.21: Increased p21^{waf} expression after treatment with PMA, TNF α /IL-1 β and IL-17A at 144 and 192 hours (based on means of duplicate wells)

4.10 ChWK cell line: EGFR and sCD44 levels after multiple treatments and comparison with IOBA-NHC line.

4.10.1 sCD44:

There was a stepwise increase in sCD44 production in response to all treatments as relative to baseline for all timepoints. The levels were much higher than those obtained from the IOBA-NHC line indicating that the ChWK line may have different capabilities in its response to injury since the confluence levels were similar for both lines. The production pattern is shown below in Figure 4.22 and the IOBA-NHC data are included for comparison purposes. Maximal production occurred in response to PMA (15ng/ml) at 192 hours and minimal after IL-17A treatment at 24 hours (5.0ng/ml). Despite different levels, the secretion profiles obtained by the two lines were remarkably similar, suggesting a common response to injury based on their morphological and functional variability. Statistical analysis was not performed given the fact the levels under comparison were produced by different cell lines under different conditions on different days.

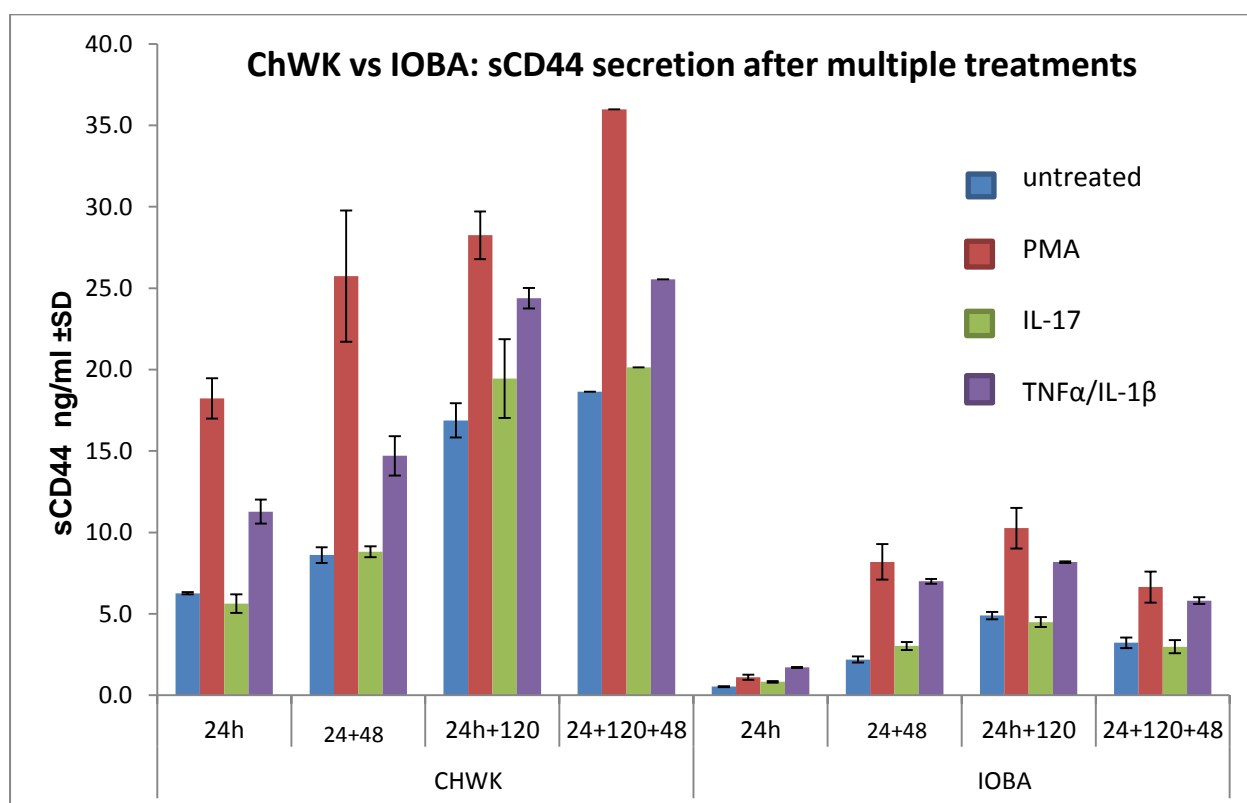


Figure 4.22: sCD44 levels in ChWK and IOBA-NHC lines after three treatments over 192 hours. Data is shown as means ± SD from triplicate wells

4.10.2 EGFR secretion

Once again there was increased EGFR secretion with higher levels than for IOBA-NHC. Maximal production occurred at 192 hours after three treatments with TNF α /IL-1 β (0.5 ng/ml). The production pattern increased with time whereas in the IOBA-NHC line there was a stepwise increase. The results are shown below in Figure 4.23 with the IOBA-NHC line data included for comparison purposes.

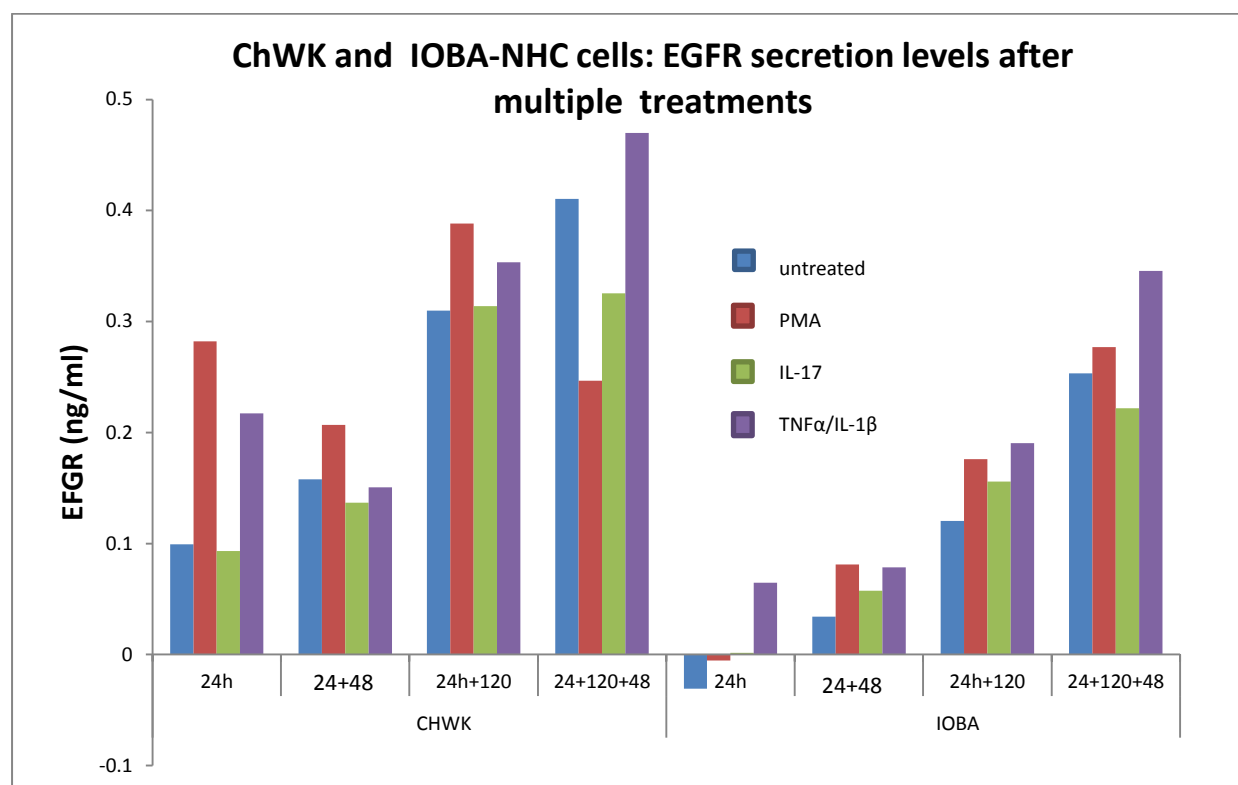


Figure 4.23: Mean EGFR secretion levels by ChWK and IOBA-NHC cells after three treatments based on duplicate wells.

4.10.3 p21^{waf} expression

All levels were increased with increasing time with maximal expression at 192 hours after PMA treatment. The untreated group also showed increased p21 expression indicating cell turnover in culture medium. All treatments induced higher levels at 192 hours compared to samples at 144 hours. The results are shown in Figure 4.24 below based on means duplicate wells.

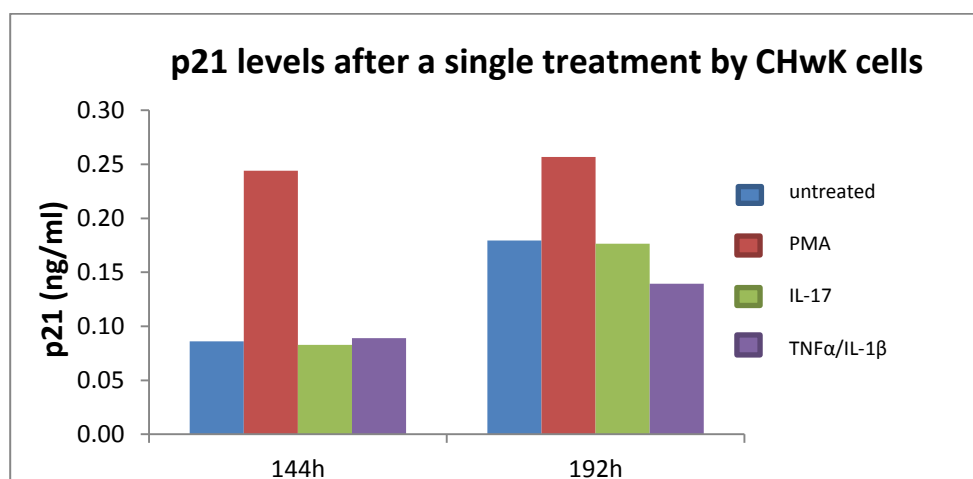


Figure 4.24: p21 levels by ChWK epithelial cells at 144 and 192 hours by duplicate wells.

The following table shows a summary of the different responses observed in p21 secretion by the two different cell lines. All treatments induced increased production of p21 by IOBA-NHC cells but not CHwK cells. This could be explained by the differences in the gene expression profiles compared to primary conjunctival epithelial cells.

Effect on p21 expression after a single cytokine treatment over 192 hours by IOBA-NHC and CHwK cells		
cytokine	IOBA-NHC cells	CHwK cells
PMA	↑	↑
IL-17A	↑	–
TNFα/IL-1β	↑	↓

Table 4.6: Summary of the effects of different cytokines to p21 expression by two conjunctival epithelial cell lines.

4.11 Effects of steroid dexamethasone pre-treatment and IL-25 to the secretion of EGFR and VEGF by IOBA-NHC cells.

Based on the previous results obtained, further studies were conducted to investigate the effect of pre-treatment with the topically administered steroid drug dexamethasone, frequently used in the management of CAED, on the secretion of EGFR and VEGF. Initially, various concentrations of dexamethasone were used and production of VEGF and EGFR assayed by means of ELISA in supernatants harvested at 48 hours. Furthermore a dose response effect to EGFR and VEGF secretion was investigated using IL-25 (or IL-17E) with and without dexamethasone pre-treatment. All sets included an untreated group of cells. Triplicate wells were used and repeated twice. The cells were treated with PMA, IL-17A, TNF α /IL-1 β and IL-25 (1 ng/ml, 10 ng/ml, 100ng/ml, 1000 ng/ml). The results are shown in Figures 4.25 and 4.26. Standard curves for all studies were obtained.

In the first set of *in vitro* studies investigating a possible dose response effect for dexamethasone EGFR was not secreted. This could be for a number of reasons: an option is that EGFR was not secreted by the untreated cells and dexamethasone influenced its secretion in the remaining groups of cells treated with cytokines and inhibited its secretion. Another explanation could be that EGFR levels were too low to detect in this instance. On the other hand, VEGF secretion was increased in all wells with all treatments and the addition of dexamethasone did not induce a reduction of levels compared to the wells without steroid pre-treatment. There was no VEGF production for the untreated cells and maximal VEGF secretion was noted after TNF α /IL-1 β treatment with 0.1 μ M dexamethasone. The production of VEGF by IOBA-NHC cells is shown in Figure 4.25.

A further set of *in vitro* studies was performed in an attempt to investigate the secretion profile of EGFR and VEGF after treatment with various concentrations of IL-25 as shown in Figure 4.26 with and without dexamethasone pre-treatment. A group of untreated cells was included as well. Supernatants were harvested at 48 hours from 4 wells for each molecule and it was repeated twice. In the analysis shown in Figure 4.26 the EGFR outlier values were not included. There was increased production for VEGF with only a small reduction in response to dexamethasone pre-treatment. The results are summarised in Table 4.7. There were

similarities in the production levels induced by the various concentrations of IL-25 in both groups and maximal secretion occurred when treated with TNF α /IL-1 β without dexamethasone (1000 pg/ml). EGFR was minimally secreted in most groups in the set without dexamethasone but maximal secretion occurred in the untreated cells (380 pg/ml). However EGFR production in the set with dexamethasone pre-treatment appeared to be reduced in the group treated with PMA, IL-25 (1.0nm/ml), IL-17A, but increased by the untreated cells. The cells treated with TNF α /IL-1 β showed minimal secretion when dexamethasone was added. When dexamethasone was added to cells prior to exposure to IL-17A, and TNF α /IL-1 β , VEGF secretion was shown to be slightly reduced or similar to levels from untreated cells suggesting that response to IL-17A and pro-inflammatory cytokines TNF α /IL-1 β may be inhibited by a steroid sensitive pattern but only to a limited degree. Given the variable secretion patterns and nature of cytokine microenvironment it is suggested that further *in vitro* experiments are performed to investigate the immunomodulatory role of dexamethasone over more time points rather than one which was used in this study.

Effect on VEGF secretion after a single cytokine treatment at 48 hours by IOBA-NHC cells		
cytokine	Without Dexamethasone	With dexamethasone
PMA	↑	↓
IL-17A	–	–
TNFα/IL-1β	↑	–
IL-25 (1 ng/ml)	↑	↑
IL-25 (10 ng/ml)	↑	–
IL-25 (100 ng/ml)	↑	–
IL-25 (1000 ng/ml)	–	–

Table 4.7: Summary of the secretion of VEGF in response to various cytokine treatments with and without dexamethasone pre-treatment. IL-25 increased the production but there was not a dose response effect. Dexamethasone did not induce a significant reduction in VEGF secretion.

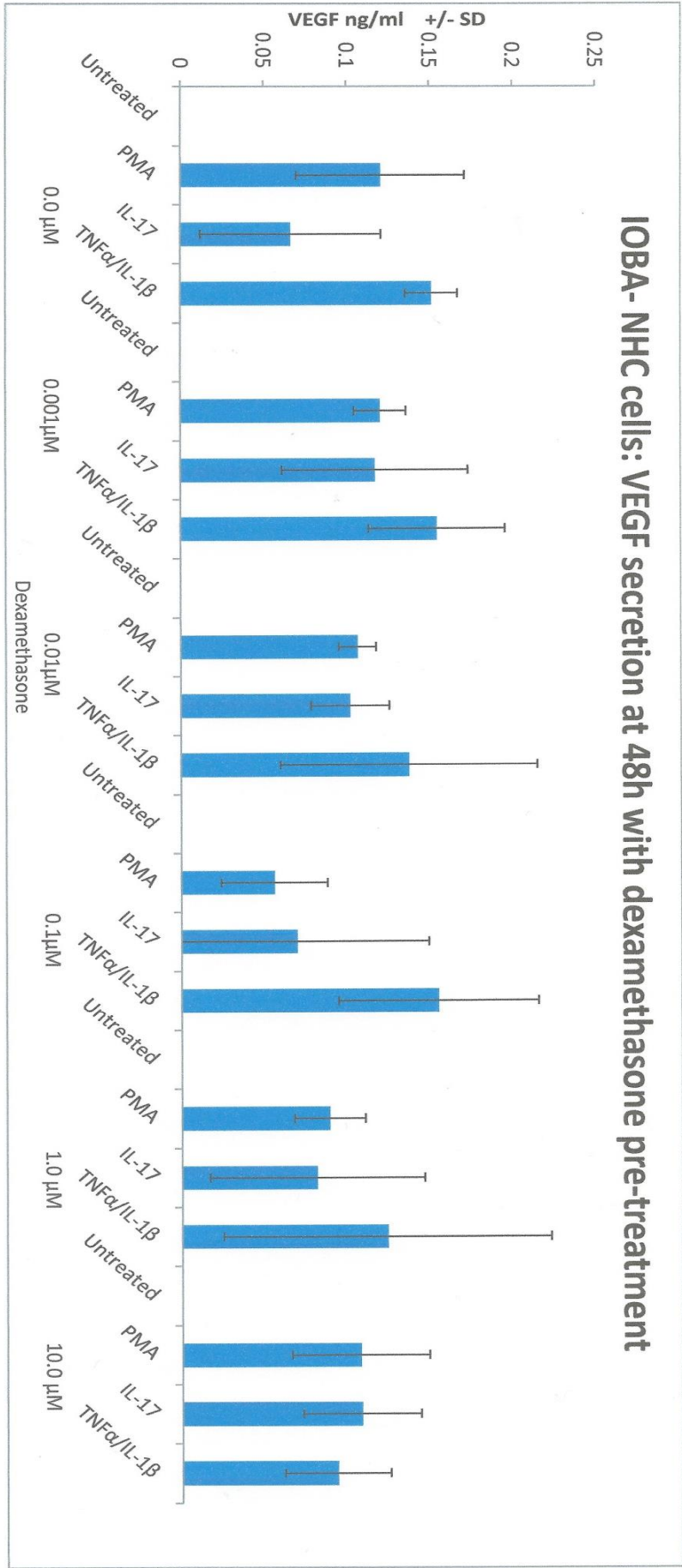


Figure 4.25: VEGF secretion after treatment with various cytokines and pre-treatment with dexamethasone at various concentrations at 48 hours.

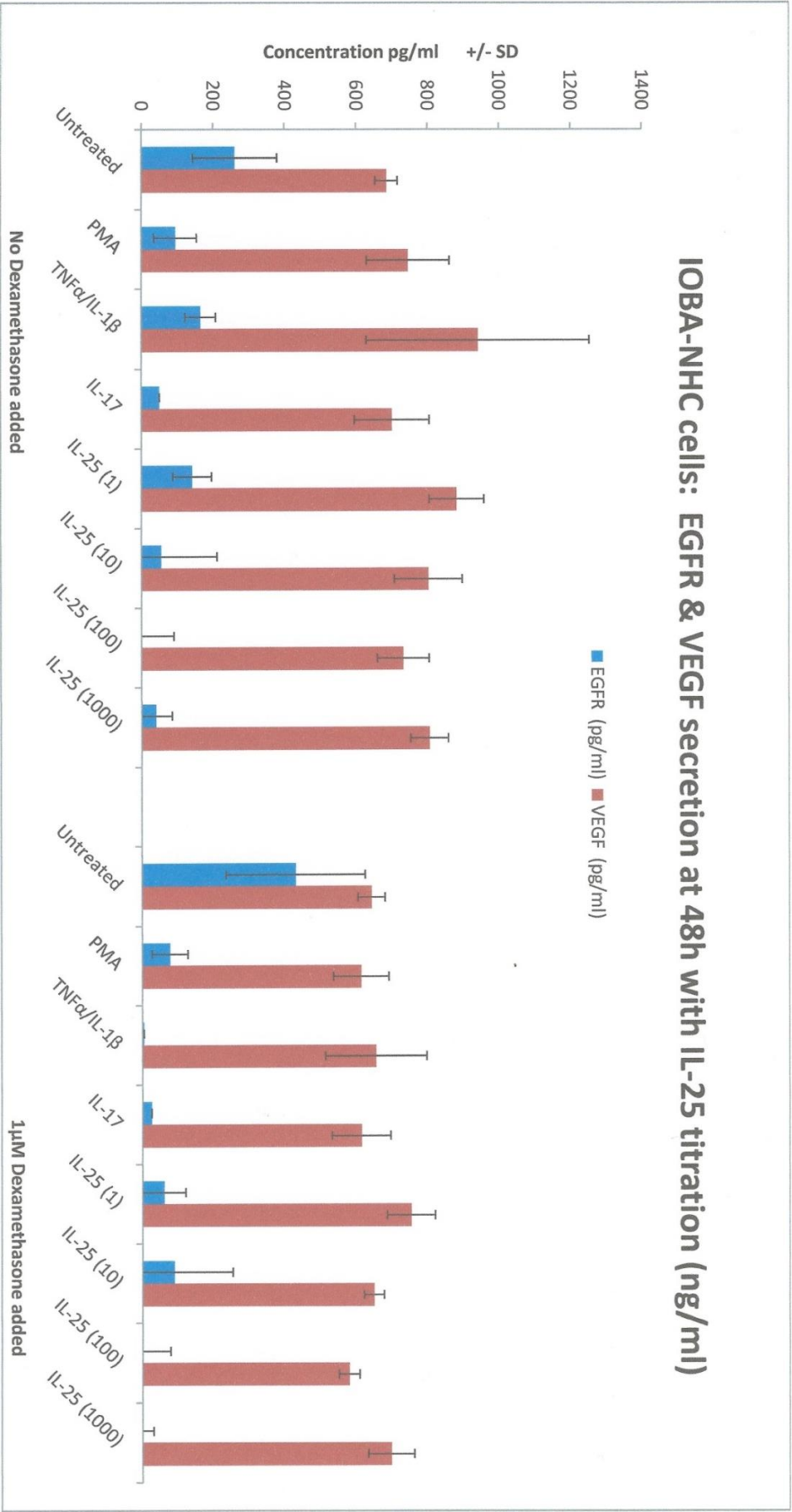


Figure 4.26: Secretion of EGFR and VEGF by IOBA-NHC cells after 48 hours with various cytokine treatments with and without dexamethasone pre-treatment.

4.12 Discussion

4.12.1 IOBA-NHC and ChWK conjunctival epithelial cell lines secrete EMTU associated molecules after treatment with pro-inflammatory cytokines and IL-17.

To date there is no published *in vitro* data on the production profiles of human VEGF-A, EGFR, sCD44, TGF α , p21^{waf} by human conjunctival epithelial cells. This is the first study to provide data on their *in vitro* secretion patterns by two conjunctival epithelial cell lines under various inflammatory conditions in response to a variety of cytokines.

Overall there is increased epithelial cell secretion for all remodelling markers after cytokine treatment which progressively increases with time in a stepwise manner. PMA and TNF α /IL-1 β induced greater secretion levels. The untreated group also showed some secretion which increased with time, reflecting an increase in cell numbers per well during the experiment, but for sCD44 and p21^{waf} there is a reduction at the final time point. The ChWK line showed increased p21 levels with increasing time. There are similar secretion patterns for EGFR and VEGF. Repeated treatments did not increase further the production levels. VEGF showed a similar secretion pattern in the single treatment pilot IOBA-NHC *in vitro* line and in the multiple treatments study. However repeated treatments did not show higher production levels. EGFR levels were lower than VEGF and similar in the two sets of experiments. Once again multiple treatments did not induce a greater increase in its levels. sCD44 acts as a positive control and an EGFR signalling enhancer and its levels also increased with time but did not show significant variations in all sets of *in vitro* experiments. Repeated cytokine/allergen treatments did not induce higher levels of secretion of the markers. All experiments demonstrated increased secretion of the remodelling markers without significant variations and no error bars were shown in levels of two experiments. The treatment with steroid dexamethasone did not alter the secretion of EGFR and VEGF *in vitro*.

This is the first study to investigate and attempt to develop an *in vitro* model of epithelial remodelling markers in ocular allergic inflammation. The *in vitro* secretion patterns of the markers chosen, based on the EMTU hypothesis in asthma, reflect the capabilities of conjunctival epithelial cell lines to respond to injury and stress.

These properties can be used to assess further their potential in repair mechanisms and the immunomodulatory roles of anti-VEGF and anti-EGFR in ocular allergy. The increased secretion levels of sCD44, EGFR, TGF α and VEGF are in agreement with the increased expression seen in the immunohistochemistry studies. So far, epithelial remodelling markers have been extensively studied in the respiratory mucosa and there is no published data to date about similar properties of conjunctival cells *in vitro*.

EGFR and TGF α production have been investigated in cultured ozone or TNF α induced cellular stress primary human nasal epithelial cells. Ozone had no effect on EGFR expression but this was not the case for TNF α (5-50 ng/ml for up to 48 hours). There was increased EGFR expression ($p < 0.01$) compared to controls in a dose-dependent manner, with maximal expression between 12-24 hours (196). EGFR positivity detected in nasal polyp epithelium correlated with the TNF α positive immunoreactive cells (254).

Primary bronchial epithelial cells and the NCI-H292 line cells were exposed to EGF and H₂O₂ aiming to achieve EGFR activation. TNF α (10ng/ml) stimulation of the line resulted in increased expression of IL-8 which correlated EGFR expression. In the same study, the authors examined the effect of dexamethasone (1.0 μ M) on EGF induced IL-8 release. Its inhibition was decreased as the dose of EGF was increased.

When serial and parallel scrape wounds were inflicted in confluent monolayers of 16HBE-14o bronchial epithelial cells, EGF addition enhanced healing and dexamethasone 0.1, 1.0, 10.0 μ M did affect wound closure (82). Additionally the involvement of EGFR activation was investigated in healing using the tyrosine kinase inhibitor tyrphostin AG1478 (a selective inhibitor of EGFR signal transduction) in H292 bronchial epithelial cell lines. It was reported that EGFR activation was an intrinsic mechanism of epithelial repair with release of an autocrine ligand. There was increased EGFR expression at epithelial sites of injury in asthmatic airways but not in control subjects. Similar evidence was provided by immunohistochemistry of airway biopsies in the same study as described in previous sections. In those asthmatic patients participating in the study using glucocorticoids there was no

significant correlation between any of the markers under investigation and steroid use (255).

Further evidence to support abnormal epithelial repair and epithelial mediated remodelling, the role cyclin dependent kinase inhibitor p21^{waf} was investigated using primary bronchial epithelial cells. Cells were exposed to H₂O₂ with or without TGFβ (10 ng/ml) and experiments with dexamethasone 1 μM were also performed. H₂O₂ was found to be a potent inducer of p21^{waf} expression. Similarly TGFβ increases p21^{waf} expression but dexamethasone did not affect its levels (98). Additionally increased asthma severity was correlated with increasing p21^{waf} expression regardless steroid use by the asthmatic subjects.

4.12.2 Advantages and limitations of IOBA-NHC and ChWK lines in experimental use

The IOBA-NHC epithelial cell line seems to exhibit features that resemble the primary human conjunctival cells in terms of morphology and function. More specifically the ChWK cells have been reported to be contaminated with HeLa cells and HeLa markers were expressed in their karyotype. Additionally they have the variant A of enzyme glucose-6-phosphate dehydrogenase which may interfere with result interpretation. However no functional similarities have been reported. Some studies have reported differences in the cytokine profiles expressed but this could be also secondary to the culture media and conditions used. These major problems associated with cell lines involving low level contamination with other cells (eg HeLa positive cells) and/or mycoplasma (although this was routinely tested for as part of our normal lab procedures), may persist undetected and cause alterations to cell behavior and gene expressions.

Both cell lines have been used before for experimental *in vitro* studies. Some researchers justify use of IOBA-NHC because it retains most of the structural and functional features of the human conjunctival epithelium. Cell lines are considered a satisfactory addition to animal studies to investigate specific cellular responses which may add to our understanding of the pathophysiological aspects of disease in humans. They permit easy and fast cell growth with small variations in experimental

conditions. Both lines have been used before by our group and others to study the role of conjunctival epithelium in allergic disease.

Cell line popularity can be estimated by the numerous publications and the American Type Culture Collection (ATCC) which includes over 3,600 cell lines from over 150 different species. Immortalized cell lines are often used in research instead of primary cell cultures since they are cost-effective, provide an unlimited supply of material and offer no concern regarding ethical issues associated to human and animal tissue experiments. However a significant drawback is that *in vitro* cell cultures are studied in the absence of their local natural environment which includes interactions with other cell types that may be important to the hypothesis being tested.

In this thesis the EMTU hypothesis of asthma is tested to conjunctival tissue and involves the interaction of epithelial cells with mesenchymal cells. Therefore a drawback of the *in vitro* experiments of this thesis is the study of EMTU markers in epithelial cells only. Co-cultures of conjunctival epithelial cells with mesenchymal cells and/or fibroblasts are suggested for further studies. Additionally, cell lines do not completely mimic primary cells. Therefore caution has been taken when designing the experiments by choosing two, rather than one, immortalized conjunctival epithelial cell lines, under conditions that other groups and our group have already tested. Further studies as key control experiments to confirm and replicate the expression patterns and properties of these markers could include the use of primary conjunctival cell lines.

The pathogenesis of chronic/persistent ocular allergy is multifactorial and despite that our understanding has increased about the role of the epithelium there are still mechanisms and functions not yet known. Therefore this adds as an additional limitation to the conclusions that can be safely drawn from the *in vitro* studies of this thesis given the artificial nature of cell lines and the fact that the markers under investigation are analysed in isolation from other factors that may also occur at the same time and not assayed.

Cell viability studies were performed to ensure that the remodelling markers' secretion was not affected by apoptosis/death. Results showed that exposure to any of the cytokines did not induce early apoptosis in comparison to untreated cells. However further studies could be carried out to ensure that production was not affected by cell density differences by means of total protein content determination (spectrophotometrical analysis of the cell lysates using the BCA assay). Alternative more sensitive way of determining total cell counts per well at the end of the experiment would be to use Trucount beads and processed by FACS to obtain an accurate live cell number per well.

Both cell lines secrete the remodelling markers assessed but the stepwise increasing secretion pattern with time and cytokine treatments by the IOBA-NHC line cells show differences in their behavior under stress conditions. In addition it is known that the gene signature expression patterns of the two lines when compared to primary conjunctival epithelial cell lines differ and that the IOBA-NHC pattern was closer to primary cultures than the CHwK (256). The results of the *in vitro* experiments provide useful information for the choice of the conjunctival cell line that is more appropriate in future experiments to increase validity of extrapolation to clinical scenarios.

CHAPTER 5

GENERAL DISCUSSION

- **Conjunctival epithelial remodelling and the application of the EMTU hypothesis in CAED.**
- **Th-17 T cell-derived cytokines may have roles in epithelial conjunctival remodelling in CAED.**
- **Re-classifying Ocular allergic disease and novel treatment strategies**

5.1 Conjunctival epithelial remodelling and the application of the EMTU hypothesis in CAED.

5.1.1. EGFR and TGF α expression parallels disease severity in CAED

The data in the present study, and from previous studies utilising VKC biopsies, supports a similar TGF β and EGFR-ligand mediated remodelling mechanism in the severe forms of disease of ocular allergy (AKC and VKC). The current study showed an overexpression in AKC and VKC of the EGFR, its ligand TGF α and CD44, known to act as the receptor enhancer, leading to its activation. The expression was mainly epithelial and was not detected in normal conjunctival biopsies. Mild cytoplasmic epithelial expression was also observed in GPC. GPC biopsies in the current study showed minimal expression of EGFR and TGF α in 2 out of 3 biopsies. There is no study to date reporting the expression levels and distribution of TGF α in the conjunctiva in chronic allergic eye disease. In CAED the EGFR expression profile from this study is in agreement with the data reported by a previous immunohistochemistry study with VKC tissue biopsies (126). In that study the investigators used an anti-EGFR mAb (1:10; Amersham) and observed expression in 8 out of 16 VKC biopsies in the deeper epithelial layers. The data from both studies may suggest epithelial mesenchymal communication and therefore may support the application of the EMTU hypothesis in CAED.

EGFR activation was investigated in corneal wound healing by means of immunoprecipitation and immunoblot analysis in an *in vivo* rat corneal model and it was concluded that EGFR was activated during corneal wound healing *in vivo* (255). It was suggested that the epithelium can produce several EGFR ligands enhancing the healing response. More specifically the level of EGFR phosphorylation was increased within 15-30 minutes after wounding and remained elevated for several hours. This timing is consistent with the initiation of the re-epithelialization stage and the data are also consistent with EFGR's role in stimulating cell proliferation in the limbus and distal to the original wound. Based on previous evidence (257-259,123) and taking into account the data of this study we may conclude that EGFR activation and its ligands' expression may indicate a possible role in the regulation of epithelial cell maintenance and repair on ocular surface epithelia. Therefore it is suggested

that in the conjunctival mucosal environment, the epithelial EGFR activation in combination with the mesenchyme may be responsible for the enhanced repair response leading to remodelling and altered tissue functions with growth factors and cytokine involvement.

TGF β epithelial expression was reported previously to be increased in VKC biopsies but was absent or very weakly expressed in control subjects (130,126). TGF β promotes fibroblast proliferation and collagen expression and has been proposed to have a role in remodelling in chronic asthma (260). The balance between TGF β mediated fibroblast differentiation and EGFR-ligand mediated cell proliferation is of particular importance in remodelling and chronic asthma pathogenesis.

The results are in agreement with other data based on immunohistochemistry studies and *in vitro* experiments involving nasal epithelial cells where increased expression of TGF α and EGF as well as EGFR in the epithelium was reported in response to exposure to ozone by means of immunohistochemistry (123). EGFR was upregulated in this acute stress model indirectly and due to the proinflammatory effects of ozone. Ten nasal biopsies after exposure to ozone showed significant epithelial EGFR expression compared to air exposure ($p= 0.007$) and 6 showed increased expression for TGF α and EGF ($p = 0.028$) (123). The data in the present study revealed higher levels of statistical significance of all markers in the severe disease ocular allergy group compared to controls despite the mild positivity observed in the GPC subjects. Furthermore treatment of human nasal epithelial cells with TNF α *in vitro*, resulted in significant EGFR secretion.

Previously, immunostaining of biopsies of asthmatic subjects exhibited strong EGFR expression. Ten normal bronchial biopsies, 13 mild and 5 severe asthmatics and anti-EGFR antibody 25 μ m/ml were used and a uniform expression of epithelial EGFR was observed and increased with disease severity (82). There was no effect of corticosteroid treatment on EGFR expression. EGFR expression patterns are in agreement with the present study but steroid drops treatment was stopped 4 weeks prior to biopsy. Given the chronicity of the disease and the need for long-term treatment it could be suggested that 4 weeks off treatment would not be long enough to influence the expression profile of EGFR and its ligands. It is therefore proposed

that in AKC and VKC, steroid treatment may not alter significantly EGFR expression patterns. The results of the *in vitro* exposure to dexamethasone which had no effect on soluble EGFR and VEGF levels would support the hypothesis that dexamethasone does not alter their cellular secretion.

5.1.2 Pro-inflammatory cytokines TNF α /IL-1 β promote *in vitro* EGFR and its ligand TGF α secretion by conjunctival epithelial cells.

The *in vitro* studies of the thesis showed increased secretion of EGFR by all groups including the untreated cells but at lower levels. Increased secretion levels were observed after treatment with PMA, then TNF α /IL-1 β and IL-17A. There was a stepwise increase in its levels with time after exposure to the recombinant cytokines but multiple treatment combinations did not produce greater secretion levels.

TNF α /IL-1 β induced increased EGFR secretion, indicating a possible role for this proinflammatory cytokine in this epithelial inflammation and repair *in vitro* model. This observation is in agreement with other reports on human epithelial cell lines. EGFR expression in human nasal epithelial cell lines (HNECs) was increased after exposure to TNF α and after exposure to ozone, and it was suggested that the later may induce an indirect effect to EGFR expression. Maximal secretion occurred at 24 hours in a dose response manner increasing gradually from 12.5 ng/ml to 200 ng/ml of TNF α (123). In this study the expression profile of EGFR was investigated over shorter and longer time periods using single or multiple cytokine treatments. Additionally further *in vitro* studies have also reported similar responses to TNF α stimulation in EGFR expression (261-263). The role of this pro-inflammatory cytokine in ocular allergy is well documented from previous studies. Immunostaining and *in vitro* results showed increased EGFR expression and secretion. PMA and IL-17A also induced EGFR expression in a stepwise manner. PMA seems to be more potent in inducing EGFR secretion than the other cytokines in the present study.

Interestingly activated neutrophils, the most prominent cell type infiltrating the airways, were reported to release TNF α in large quantities in ozone exposed human nasal mucosa (123). The results in Chapter 4 of this study have shown increased EGFR secretion by conjunctival epithelial cells in response to TNF α treatment. The

data from the current study are in agreement with previous studies conducted in nasal mucosa. It is noteworthy that the TNF α levels produced by the cell lines itself may have not been high enough to induce EGFR expression and other cell types with well-known proinflammatory roles (e.g. eosinophils, mast cells and macrophages) can also contribute. In addition it was observed that the positive staining for EGFR in nasal polyp epithelium correlated with the number of TNF α positive immunoreactive cells (264).

EGFR signalling is proposed to be an intrinsic part of the response to injury and not dependent on an exogenous ligand (82). Human bronchial epithelial cell lines were used (16HBE14o) and demonstrated *in vitro* increased secretion of TGF α , amphiregulin and HB-EGF which resulted in EGFR activation after scrape wounding of cell monolayers. Additionally it was showed that EGF promotes bronchial epithelial repair *in vitro* and EGFR expression was significantly increased in the areas of epithelial damage in bronchial biopsies of asthmatics but not normal subjects (82). TGF α secretion was also increased after exposure of human nasal mucosa to ozone and assessed by means of immunohistochemistry compared to nasal mucosa exposed to air (123).

5.1.3 EGFR and TGF α levels correlate with eosinophilic activation and neutrophilia

Notably in the severe of forms of disease AKC and VKC it was reported that there is upregulated eosinophilic activation and different cytokine profiles (159). It has been found that in VKC there was eosinophil co-expression of proinflammatory cytokines IL-3, IL-5, IL-6, GM-CSF whereas in AKC the profile changed with increased eosinophilic expression of IL-4, IL-8 and GM-CSF (18). IL-4 is known to be produced by Th2 T cells, mast cells (267) and eosinophils (154) and participates in the Th2 type response. In chronic asthmatics EGF significantly stimulated IL-8 production in a dose dependent manner (254) which is a potent neutrophil chemoattractant (265). EGF-stimulated IL-8 production was found not to be affected by steroid treatment (266). Neutrophilic infiltration is a feature of increasing severity in chronic forms of ocular allergy with highest neutrophil cell counts in AKC (154). Therefore the

epithelial damage could lead to neutrophilia via an EGFR dependent increase in IL-8 production by the conjunctival epithelium.

5.1.4 Increased CD44 levels in CAED parallel disease severity.

Previous studies have reported that CD44 production is associated with epithelial injury, can be regulated by cytokines (103) and has a physiological role in epithelial repair (104). The increased expression of CD44 in inflammatory diseases acts as a positive control confirming the degree of tissue response to injury in chronic forms of disease (AKC and VKC). However, some CD44 expression was also observed in the milder inflammatory condition GPC in the control group as well as in two normal controls hence it failed to reach significance (taking statistical significance at $p < 0.05$). The expression pattern follows that of EGFR and its ligand TGF α and is mainly epithelial with milder patchy or diffuse stromal uptake. Increased expression of CD44 reflects a response to injury of the layer involved; hence its role as a positive control reflecting tissue damage.

Both cell lines showed increased production of the positive control marker, hyaluronate receptor, CD44 in all groups, in response to pro-inflammatory cytokines, indicating a baseline degree of cell turnover, probably reflecting the culture tissue conditions. The increased levels paralleled the EGFR and VEGF secretion profiles *in vitro* which have been also shown to be overexpressed in VKC and AKC biopsies.

CD44 expression was investigated by means of antibody-staining and confocal microscopy using cultured differentiated human bronchial epithelial cells (268). CD44 was expressed in normal bronchial airway biopsies in the areas of epithelial damage, 18 hours after scraping (268). Some but not all cells co-expressed the proliferation marker Ki-67 in addition to CD44. A similar response of increased CD44 expression was found in two of the healthy conjunctival tissues included in the control group of the thesis. Overall CD44 expression levels were also shown to be increased in the bronchial epithelium of asthmatics (102).

5.1.5 Increased p21^{waf} secretion *in vitro* in IOBA-NHC conjunctival epithelial cell line parallels EGFR and CD44 profiles.

From the results of the *in vitro* studies there was increased p21^{waf} secretion after treatment with all cytokines. This observation is in agreement with other studies which suggested that the expression of this cyclin-dependent kinase inhibitor is increased in epithelial injury. p21^{waf} overexpression in asthmatic mucosa affects cell proliferation and survival leading to abnormal epithelial repair and remodelling (78).

The increased production of EGFR does not seem to be associated with increased proliferation as part of a cellular response to injury and is not associated with an increased expression of proliferating cell nuclear antigen (PCNA) (269). It is known that p21^{waf} interacts with PCNA to prevent its activation of DNA polymerase δ (269). CDK inhibitor p21^{waf} interacts with cyclins D and E to inhibit transition from G1 to S phase via inhibition of the phosphorylation of the retinoblastoma protein (269). Cell division is a highly regulated process and transition through the restriction point and entry into the S phase is regulated by CDKs that are in turn regulated by cyclins D, E and A. CDK activity is modulated by phosphorylation and requires cyclin binding and may be inhibited by the CDK inhibitory proteins (270).

Cellular population homeostasis depends on the balance between cell proliferation, growth arrest and apoptosis. Cell differentiation, growth inhibition, DNA damage are known to affect the levels of p21^{waf} which in turn affects all the processes described above (271). After treatment of primary bronchial epithelial cell *in vitro* with TGF- β , p21^{waf} expression and localisation was observed to be in the nucleus as also observed in tissue sections (98). Expression of p21^{waf} after tissue response to injury is important since it is involved in cell apoptosis and survival processes. Its anti-apoptotic role correlates with its cytoplasmic distribution in oxidant treated bronchial epithelial cells (98).

By means of immunohistochemistry the distribution and expression of p21^{waf}, proliferation marker Ki67 and EGFR was investigated in bronchial biopsies from asthmatic children. Immunostaining for Ki67 was localised only in the basal cells and its expression was significantly reduced in the asthmatic epithelium compared to normal subjects. There was a positive correlation in the increased levels of EGFR and p21^{waf} expression but there was no positive correlation between eosinophils and

p21^{waf} or Ki67 (272). Based on *in vitro* studies, epithelial injury induces expression of various growth factors (e.g. TGF β , FGF, PDGF, ET-1) and these in turn promote fibroblast proliferation (50,272). This effect can be further enhanced by inhibition of EGFR mediated epithelial repair (82). These results support the suggestion that abnormal epithelial mesenchymal communication in the injured mucosal membrane mediate remodelling processes and disease perpetuation (97).

Therefore increased p21^{waf} expression in conjunctival epithelial cells may suggest that in chronic ocular allergic disease there is an abnormal epithelial response to injury leading to reduced cellular proliferation and replacement of injured cells. Furthermore this suggests an imbalance in the relationship between cellular apoptosis and survival affecting the regulation of conjunctival epithelial cell population and homeostasis. These results are in agreement with the observations made in chronic asthma and may contribute to disease perpetuation and epithelial mediated remodelling in AKC and VKC.

5.1.6 TNF α /IL-1 β and IL-17A induce VEGF secretion by conjunctival epithelial cells *in vitro*.

It has been demonstrated that fibroblasts and monocytes stimulated *in vitro* with IL-17A produce VEGF-A (273,274) and *in vivo* cornea after HSV infection via induction of IL-6 expression (305). Additionally IL-6 was shown to be a regulator of IL-17A production by T cells (275,276). Increased ocular surface IL-6 production has been documented in human dry eye disease (277,278) and there was also a correlation with disease activity (279). VEGF has a well-established role in angiogenesis and is also known to participate in remodelling amplifying the continuation of the chronic inflammatory process (38,280). Its role in epithelial mediated remodelling in mucosal inflammatory disease has been suggested with specific reference to chronic asthma (260) and vernal keratoconjunctivitis (281,126).

The *in vitro* results of the current study on VEGF secretion profile are in agreement with the findings by other studies utilising VKC biopsies (281,126) suggesting that conjunctival epithelial mediated remodelling plays an important role in the immunopathogenesis of CAED. However it is not yet known if VEGF production occurs secondary to EGFR upregulation or is indirectly mediated via cytokines acting as intermediate mediators. The roles of the pro-inflammatory cytokines TNF α and IL-17A may suggest that intermediate factors participate in disease perpetuation via growth factor expression.

Growth factors are well known to regulate production of the components of extracellular matrix, integrins and collagens. The epithelium in the present study seems to be the source of VEGF and similar studies have documented that TGF β , bFGF and PDGF are derived from the conjunctival epithelium in VKC (126,130) as well as fibroblasts and other inflammatory cells participating in the cell mediated immune mechanisms in VKC (282,283).

5.1.7 Epithelial mediated remodelling in asthma and chronic allergic ocular disease

EGFR overexpression has been documented in asthmatic epithelia at both the mRNA and protein levels (82,83,284). Additionally *in vitro* studies reported that EGFR and its ligands play an important role in epithelial repair in asthmatic airways (82). More specifically the *in vitro* studies performed in bronchial epithelial cell cultures suggest that EGFR plays an important role in bronchial epithelial repair and remodelling in asthma (82). The significant upregulation of EGFR expression observed in the current study in VKC and AKC biopsies is in agreement with the findings from other studies in airway mucosa and VKC as described above. This suggests that abnormal epithelial repair is participating in conjunctival epithelial remodelling in the chronic forms of ocular allergic disease as proposed by the EMTU hypothesis in chronic asthma.

VEGF is also overexpressed by the conjunctival epithelium as documented by immunohistochemistry in VKC biopsies (126), flow cytometry in tears of VKC patients (281) and *in vitro* in the current study as described previously. In asthmatic airways there is increased VEGF expression which is also produced by eosinophils and macrophages (285). This observation was associated to the increased vascularity observed of the bronchial mucosa and the numbers of VEGF-positive cells (285). In a murine model of asthma there was increased VEGF expression and furthermore an increased VEGF expression in asthmatic airways was detected after antigenic stimulation (286,55).

The factors described above seem to have a central role in epithelial remodelling in chronic asthmatic mucosa and in severe forms of ocular allergic inflammation. Additionally fibrogenic growth factors PDGF, bFGF, TGF- β 1 which are known to participate in remodelling were all found to be increased in the epithelium in VKC compared to normal subjects (126,130) and they have been reported to promote fibroblast proliferation and increased extracellular matrix production and deposition in the pulmonary mucosa in pulmonary fibrosis (59). It has been suggested that binding of all of these factors to the extracellular matrix glycosaminoglycans may induce prolonged activity of the factors at the fibrotic areas (130). The conjunctival epithelial cells communicate via cytokines and growth factors with the fibroblasts and regulate

cellular processes, wound healing and angiogenesis (51,52). Overexpression of PDGF, bFGF and TGF- β 1 has been demonstrated by bronchial epithelial cells in a three-dimensional *in vitro* model and promote fibroblast proliferation (50).

Conjunctival remodelling is known to affect VKC and results from this thesis suggest that remodelling markers are upregulated in CAED. However no studies to date have demonstrated the histological changes associated with remodelling in the conjunctiva. Therefore this is a limitation of the current study and it is suggested that if more biopsies were available, more histopathological features could be investigated in AKC and VKC compared to controls. More specifically, using conjunctival sections stained with haematoxylin and eosin, measurements of basement membrane thickening or fragmentation and areas of destruction of conjunctival epithelium could be assessed as in previous studies in asthmatic airways (287-289) and an attempt to relate the findings with the epithelial conjunctival expression of EGFR and TGF α .

In the asthmatic lung there is growing evidence over the last few years that the epithelium plays a crucial role in the immunopathogenesis of airways remodelling. An emerging theory proposed an inability of the airways epithelium to properly repair leading to EMTU activation and remodelling. More specifically it proposed that Th2 cytokines co-participate with the epithelial damage leading to inappropriate reactivation of the EMTU, inflammation and remodelling in chronic asthma. This thesis investigated the possibility of applying the EMTU hypothesis of asthma to CAED.

Key EMTU molecules are found to be upregulated by conjunctival epithelial cells but these findings are mainly *in vitro* investigations in isolated epithelial cells. The immunostaining data, although they provide evidence that EMTU molecules are overexpressed in CAED, on their own are not sufficient to support fully the application of the EMTU hypothesis in CAED. Although this is the first study to look for evidence on the EMTU hypothesis in the conjunctiva and CAED, the interactions between the different cell populations forming the EMTU have not been studied either *in vitro* or *in vivo*. Therefore future challenges include the development of systems that will enable this hypothesis to be fully tested and the hypothesis that inherited susceptibility genes e.g. ADAM33, influence the EMTU reactivation and

upregulation of molecules and mediators indicative of the remodelling processes in CAED.

This study focused on *in vitro* experiments with isolated conjunctival epithelial cells treated with recombinant proteins. One mouse model has been recently developed in severe AED (324) but there have been no developments on an animal model for CAED. Therefore fibroblast-epithelial co-cultures of primary conjunctival cells with cell junction formation and plated on collagen/matrix membranes to develop three-dimensional cultures or interaction of cells, are suggested for future laboratory work. These models may retain the advantages of isolated cell cultures but also allow investigations of long-term events and studies of the pharmacological targets of intercellular processes involved in CAED. A CAED mouse model has been recently developed which will hopefully enable many of the *in vitro* experiments to be performed *in vivo* to investigate further the application of the EMTU hypothesis and future treatment strategies. Briefly, murine dendritic cells are pulsed with short ragweed pollen (SRW) and then injected subconjunctivally into SRW-sensitised mice which are then topically challenged daily with SRW for 5-7 days. Signs and symptoms of a clinically severe form of allergic conjunctivitis were observed (338).

Compared to airway remodelling, where there is growing interest and amount of investigations behind the mechanisms involved, conjunctival remodelling and CAED pathogenesis have not been extensively investigated. Questions remain not fully answered and it is hoped that the results of this thesis will support future work towards understanding better the following elements:

- 1) Is conjunctival epithelial remodelling the cause of CAED or a product of chronic inflammation?
- 2) Is inflammation essential for the development of conjunctival epithelial remodelling or they are both parallel events contributing to disease pathogenesis?
- 3) Is conjunctival epithelial remodelling in CAED reversible?
- 4) Is conjunctival epithelial remodelling genetically determined?

Although conjunctival remodelling is considered to occur as a consequence of chronic inflammation, the idea that the EMTU reactivation in chronic asthma induces remodelling has led to the suggestion that inflammation and remodelling are parallel than sequential events. The results of this thesis have shown for the first time that there is upregulation of remodelling associated molecules and mediators related to the EMTU in CAED. This hypothesis may explain in part why the prolonged use of steroids may have a limited effect on limiting disease progression in CAED.

5.1.8 EGFR and associated epithelial remodelling mediators in CAED are not expressed in OCP

In the present study there was no expression of EGFR and its ligand in active and chronic OCP. This may suggest separate T cell driven responses in fibroblast-mediated remodelling mechanisms in OCP differentiating this conjunctival inflammatory disease from an epithelial-mesenchymal mediated remodelling mechanism with EGFR activation in chronic allergic disease. Data reporting overexpression of collagens (130), metalloproteinases (130,281), growth factors (TGF- β , FGF, PDGF, VEGF) (130,131,126,281) in VKC as opposed to controls, contribute to remodelling. This evidence taken together with the overexpression of EGFR and its ligand may support the role of epithelial-mesenchymal mediated communication leading to remodelling, disease perpetuation and chronicity.

Given the absence of EGFR expression and its ligand in active and chronic OCP it is likely that EGFR mediated remodelling of the conjunctiva is not a feature of autoimmune disease. Hence the immunostaining data of this thesis suggest that there is no evidence to support the application of the EMTU hypothesis to OCP. This re-enforces the fact that OCP is a different disease process where cicatrizing autoimmunity against the basement membrane is the key mechanism rather than remodelling mediated by epithelial injury. The cicatrizing component of the disease leads to blindness due to surface failure, corneal vascularization and opacification. EMTU activation instead is a feature of epithelial-mesenchymal driven response in chronic forms of mucosal inflammatory disease with an allergic component in its immunopathogenesis. Therefore, cellular and cytokine responses together with EGFR mediated interactions may suggest that there are several self-propagating vicious cycles contributing to disease perpetuation and chronicity.

In OCP, progression of sub-epithelial conjunctival fibrosis due to continuous excessive matrix deposition leads to conjunctival contraction, forniceal shortening, symblepharon, meibomian gland dysfunction. Various molecules participate in the fibrotic process and so far most research reports investigated the role of conjunctival fibroblasts. T cells are increased in the subepithelial infiltrate at all phases of ocular pemphigoid disease. TNF α expression is increased by a large number of stromal

infiltrating cells in active OCP. TNF α induces increased MMP-9 expression and decreased TIMP-2 as well as increased ICAM. TNF α seems to have a profibrotic, chemoattraction and pro-inflammatory roles in human conjunctival fibroblasts (337). However the results of this thesis suggest that a concomitant EGFR upregulation is not observed suggesting that if TNF α is responsible it may be acting indirectly via activation of another pathway.

5.1.9 EGFR expression is not affected by steroids in severe asthmatic mucosa.

It has been reported that EGFR expression and neutrophilia in severe asthma are refractory to corticosteroid treatment (82,293). More specifically the synthetic corticosteroid dexamethasone (1 μ M) did not affect basal or EGF-stimulated wound closure (82). The same applies when used at 0.1, 1.0 or 10.0 μ M concentrations but not for 100 μ M for which there was significant inhibition. It was suggested that the result regarding the 100 μ M dose may reflect a non-specific effect on wound closure given this very high steroid dose. In the current study it is also observed that there is no significant effect of the steroid dose on levels of EGFR secretion after cytokine treatment. Steroids induce increased p21^{waf} expression (294) but here was no obvious relation between corticosteroid dose used by severe asthmatic subjects and the levels of bronchial epithelial p21^{waf} expression (98).

A repeat wound model has been described using mucociliated human bronchial epithelial cells to investigate the effect of therapeutic agents in epithelial repair in a chronic state. It was reported that epithelial repair is dependent on autocrine EGF-mediated migration. Dexamethasone induced an inhibitory effect in repair and cellular proliferation at the short-term but showed increased long-term potential. However the mechanism to explain this effect remains unclear (295).

All the above indicate that steroids used in the treatment of chronic forms of asthma and severe ocular allergy, do affect the expression of mediators involved in remodelling which are accountable for disease irreversibility and progression. Additionally steroids have no effect on cellular homeostasis which is regulated by cell proliferation, growth arrest and apoptosis. Taken all together it can be concluded that the cellular changes in the mucosal epithelium of the bronchial airway and conjunctiva in severe inflammatory disease are not regulated by steroids. This may explain why steroids are ineffective in achieving disease control in the severe forms of these inflammatory diseases of the mucosal membrane such as asthma and CAED.

5.1.10 Epithelial-mesenchymal transition (EMT): repair and fibrosis

The roles of mesenchymal cells in tissue repair, fibrosis as well as tumour metastasis are not fully understood. It is suggested that epithelial-mesenchymal transition (EMT) is an important source of these cells and is a biologic process that enables a polarised epithelial cell to transform into a mesenchymal cell with ECM production and migratory properties, invasiveness as well as resistance to apoptosis (296). There are three types of EMT representing distinct biological processes: Type 1 occurs in embryogenesis and mesenchymal cells generate secondary epithelia but do not cause fibrosis; type 2 EMTs that occur with wound healing; type 3 EMTs that occur in neoplasia. Type 2 EMTs are associated with inflammation, wound healing and regeneration generating fibroblasts in an attempt to repair tissues after trauma or inflammatory injury (297).

EMTs are found to occur in fibrosis in the kidney, liver, lung and intestine (298-301). Markers of fibrosis secreted by mesenchymal cells in EMTs include, fibroblast specific protein 1 (FSP1) which is a cytoskeletal protein, α -SMA (α - smooth muscle actin), collagen I, vimentin and desmin (302, 294). TGF β is shown to induce EMT-mediated fibrosis (302, 303). Recombinant BMP-7 is known to act as an endogenous inhibitor of TGF β induced EMT (302, 10) and was shown to reverse EMT with repopulation of healthy epithelial cells in mice with fibrosis when administered systemically (303). EMT evidence was reported in kidney biopsies with fibrosis in humans using labelling of vimentin, cytokeratin and α -SMA (304) with similar observation made in intestinal biopsies in patients with Crohn's disease (305).

Based on *in vitro* experiments, EMT occurs in the repair of the lacrimal gland and is responsible for the generation of mesenchymal cells as suggested by the expression of EMT markers vimentin and snail1, at the peak phase of the lacrimal gland repair process and in mesenchymal cells isolated from injured lacrimal glands. The authors suggested that by reinforcing our understanding of the EMT molecular processes it would enable researchers to propose novel therapeutic strategies that target lacrimal gland healing and repair and promote production of the aqueous layer of the tear film (306). All the above may suggest that new treatment strategies by inhibiting the EMT processes may have a role in the management of the severe chronic forms of inflammatory disease.

5.1.11 Summary

In summary there is immunohistochemical and *in vitro* evidence that epithelial remodelling markers are overexpressed in the conjunctival in allergic conditions. Combining the EMTU hypothesis for the pathogenesis of severe asthma and the results presented in this thesis it is suggested that a similar paradigm to asthma EMTU occurs in chronic and persistent forms of ocular allergy which may account for disease progression and severity. Figure 5.1 below (modified by 260) attempts to summarise the events in AKC and VKC and present a conjunctival EMTU paradigm to account for the severity and chronicity of these forms of disease.

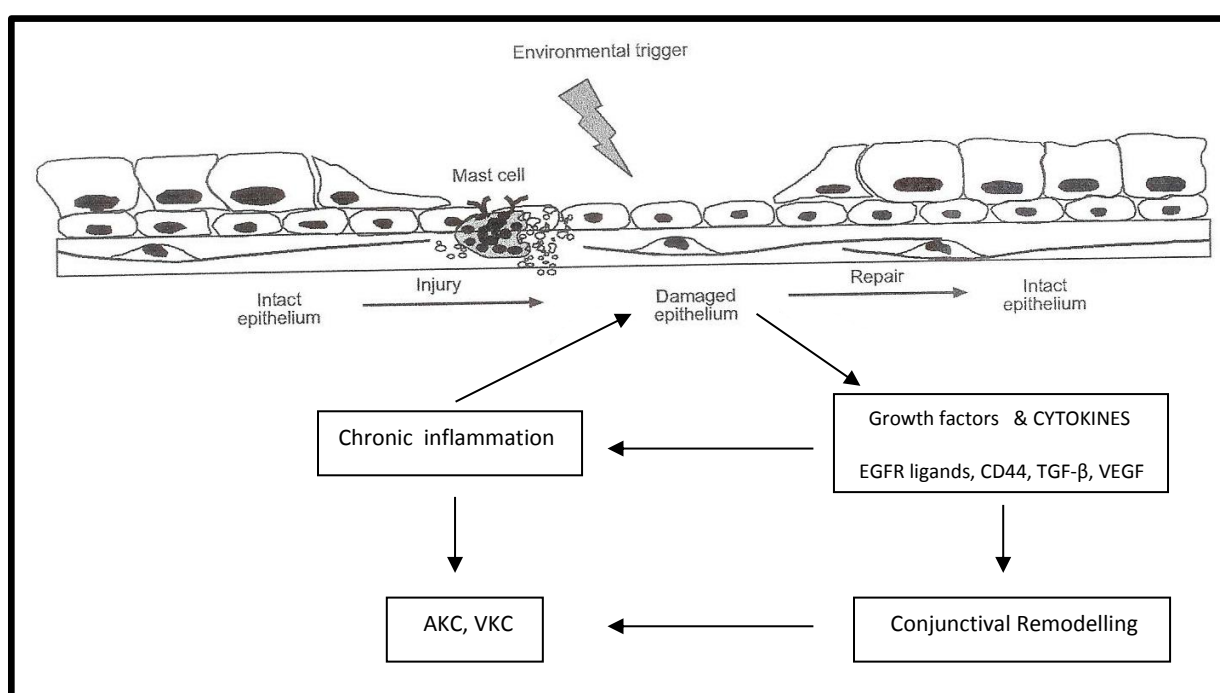


Figure 5.1: A proposal of the application of EMTU hypothesis in CAED based on the results of this thesis (modified by 260).

Based on evidence from previous studies on remodelling mediators in VKC (126,130) and the results of this study on the role of Growth factors and epithelial remodelling is suggested that conjunctival epithelial remodelling co-participates in disease pathogenesis and perpetuation. In addition to the mediators, cytokines and cellular aspects as shown in Table 5.1 below (modified from 136), growth factors participating to tissue remodelling have been added in an attempt to update the already known evidence so far. An updated table summarising together all cellular and molecular participants is shown below.

OCULAR ALLERGY	IgE mediated (SAC/PAC)	IgE and non-IgE mediated (VKC/AKC)	Non-IgE mediated (GPC)
Mediators	Histamine Prostaglandine D2 Leukotrienes PAF ICAM	Histamine Prostaglandins Leukotrienes PAF ICAM, VCAM, MMPs, proteases	Prostaglandins Leukotrienes ICAM
Remodelling Factors		TGF β , TGF α , PDGF,EGF, EGFR, VEGF, CD44	CD44, EGFR, TGF α
Cytokines/chemokines	IL-1, IL-4, IL-8, TNF α , IFN γ	IL-1, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17, TNF α , IFN γ , Eotaxin, MCP, RANTES	IL-1,IL-6,IL-8,TNF α , IFN γ , RANTES, MCP
Cells	Mast cells Neutrophils Eosinophils	Mast cells, T cells, NKT Neutrophils Basophils, Macrophages	T cells Neutrophils Macrophages

Table 5.1: A summary of the cellular and cytokine participation in ocular allergy including the results of this Thesis.

5.2 Th-17 T cell-derived cytokines may have roles in epithelial conjunctival remodelling in CAED.

5.2.1 IL-17A induces EGFR secretion *in vitro* by human conjunctival epithelial cells

T cell cytokine profiles differ in CAED with a Th2 type pattern (IL-3, IL-4, IL-5) in VKC and Th1 type (IL-2, IFN γ) in AKC (159). It may be uncertain whether further T cell variations may be implicated in chronic allergic disease forms. The presence of Th17 cells in ocular allergic disease was recently investigated in CAED (244). More specifically there were increases in epithelial and subepithelial Th17 (IL-17 producing CD4⁺ T cells) cells in AKC and VKC conjunctival biopsies compared to controls (normal biopsies and SAC). Together with the detection of increased levels of IL-17E in tears specimens from VKC subjects, the data suggest a pro-inflammatory role for Th17 cells. IL-17 expressing cells have also been reported in autoimmune dry eye in man and in a mouse model (291) and in OCP (292). It is likely that IL-17 may have different roles in various forms of ocular surface inflammatory diseases. Further studies are required to investigate the role of IL-17 in the pathogenesis of tissue remodelling.

Six members of the IL-17 cytokine family are secreted by activated memory T cells. IL-17 has been shown to induce reduced expression of β -defensin which has antimicrobial properties indicating a role of IL-17 in antimicrobial defence (287). IL-17 which is known to have a pro-inflammatory role is shown to induce IL-8 production in human bronchial epithelial cells which in turn promotes neutrophil migration (32). IL-17A induced neutrophil infiltration is also observed in cornea after HSV infection (287).

Additionally the increase in neutrophils was positively correlated to increased EGFR expression in the nasal epithelium after exposure to ozone (123). The same study showed that exposure of nasal epithelial cells to ozone induced IL-8 production. Increased IL-8 production *in vitro* occurred after treatment of bronchial epithelial cell lines (H292) with H₂O₂ and EGF (254). More specifically EGF induced a dose-dependent IL-8 release. In the same study there was positive correlation of IL-8 and EGFR expression with neutrophil numbers (264).

These events in addition to the cytokine effects from other cell types involved, participate in disease perpetuation re-enforced by abnormal epithelial mediated remodelling in chronic mucosal inflammatory disease and continuous response to injury.

Exposure of human conjunctival epithelial cells to IL-17A induced IL-8 and CD44 expression as well as MMP2 and MMP-9 (244,287). Additionally, it was shown that treatment of human corneal epithelial cells with IL-17A was shown to induce increased expression levels of MMPs mRNA and impaired barrier function (167). Similar observation supported that IL-17A promotes MMP-2, -7, -9 and in addition it affects VEGF-A bioavailability (307). In this current study it was found that IL-17A induces increased EGFR secretion by conjunctival epithelial cells *in vitro* after a single or multiple treatments but it is not clear if this effect is direct. It is also shown that this effect is not sustained and was reduced after 48 hours. It is therefore suggested that other factors and cytokines may participate as intermediaries. It is proposed that IL-17 via its proinflammatory and regulatory actions may have roles in promoting conjunctival epithelial remodelling in ocular allergy.

5.2.2 IL-25 (IL-17E) induces VEGF secretion *in vitro* by conjunctival epithelial cells with and without dexamethasone pre-treatment.

It has been reported that IL-25 (or IL-17E) amplifies the Th2 cell-mediated inflammation in asthma (256-260) but its role in conjunctival allergic response remains unclear. IL-25 with TSLP (Thymic Stromal lymphopoietin) promotes Th2 differentiation and proliferation resulting to enhanced Th2 functions with increased expression of IL-5 and IL-13. IL-25 enhances Th2 expansion and sustains Th2 cell activation (308). TSLP is an IL-7-like cytokine that can activate dendritic cells (DCs) which, in turn, can re-enforce the allergy-inducing properties of Th2 cells by upregulating the expression of pro-allergic genes (309).

Th2 cytokines are known to participate in VKC (36,218,310) and more specifically Th2 cytokines IL-4, IL-5 were found to be significantly elevated (311-313,151,281). Given also that in VKC, there is increased expression of growth factors VEGF, TGF β , PDGF, EGFR (130,126,281) it may be suggested that IL-25 may have a role

in influencing their overexpression indirectly. Hence it is likely that IL-25 may have a role in augmenting the inflammatory response in the severe forms of ocular allergy leading to disease perpetuation and epithelial mediated remodelling as suggested by the EMTU hypothesis.

However in the current study there was not a dose response effect in VEGF and EGFR levels after treatment with increasing concentrations of IL-25. Additionally at 48 hours the EGFR expression is minimal (at pg/ml) and hence with possible little clinical relevance. On the contrary VEGF secretion was elevated but there was no significant difference in its levels with increasing IL-25 concentrations. The EGFR levels at 48 hours after IL-25 treatment in the current study were lower, in both groups (with and without dexamethasone) including the untreated cells. Given from the results of other *in vitro* experiments in the current study, EGFR levels increased with increasing time. For the IL-25 treated cells, sampling took place once at 48 hours and this could provide a possible explanation for the low levels observed. On the contrary VEGF expression is greater. This may be explained by the fact that epithelial VEGF production may be influenced directly by other factors. On the contrary, EGFR's secretion may be indirectly influenced by intermediate cytokines interfering with the epithelial responses to inflammation in the cytokine microenvironment.

VEGF levels appeared to be greater than EGFR's and steroid pre-treatment did not cause significant reduction in its levels. This observation is in agreement with the reports from other studies which investigated the effect of steroids to EGFR expression. EGFR's levels paralleled IL-8 expression and neutrophil numbers in the submucosa in asthmatic bronchial biopsies. In the patients using corticosteroids there was no reported correlation between any of the measured parameters and steroid dose (254). These findings provided evidence to support that EGF stimulates IL-8 mRNA production from bronchial epithelial cells linking EGFR upregulation and neutrophil response. In conjunctival epithelial cell lines PMA, TNF α /IL-1 β and polyI:C (TLR3) induced IL-8 expression (244). Figure 5.2 below summarises the findings of the thesis and presents a proposal for the possible role of proinflammatory and Th17 cytokines along with EMTU mediated remodelling in CAED.

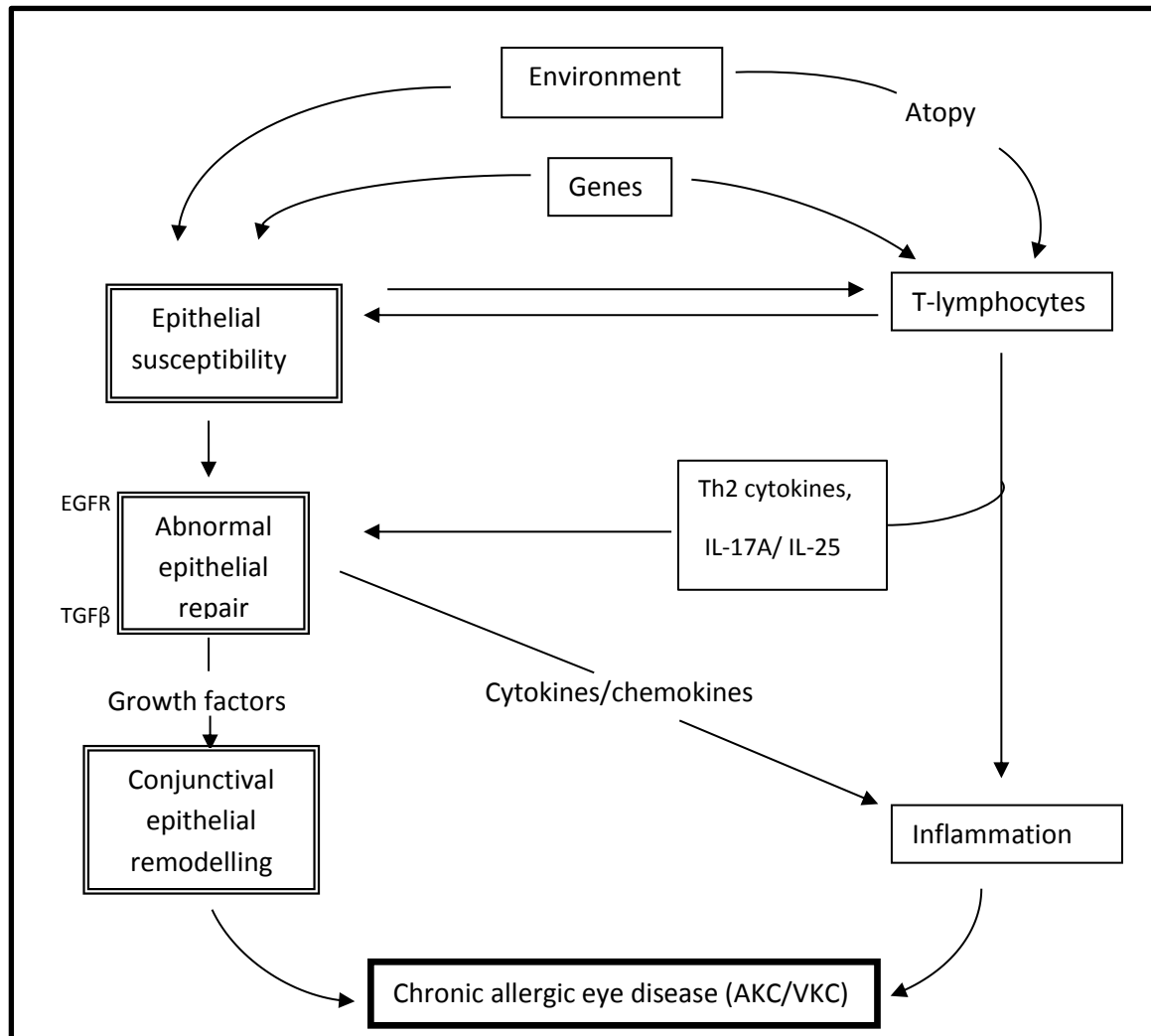


Figure 5.2: A proposal for the interactions between Th2/Th17 mediated inflammation and the EMTU in CAED pathogenesis (Modified by reference 84).

5.2.3 Toll Like receptors (TLR) and conjunctival epithelial cells

Toll pattern recognition receptors play an important role in the innate immune system and are believed to function as dimers. They recognize ligands of microbial origin and are present on various cell types including dendritic cells and provide a link between adaptive and innate immunity. After binding to the ligands the pathogen may be phagocytosed and digestion products can be presented to CD4⁺ T cells. Immune cells may produce cytokine mediating inflammatory responses. Recent evidence regarding the roles of TLRs expressed by epithelial conjunctival cells indicates potential new immunomodulatory roles of the epithelium in inflammatory responses of allergic origin. LPS is TLR4 ligand and zymosan binds TLR2.

In contact dermatitis, nickel (Ni²⁺) triggered an inflammatory response by directly activating human Toll-like receptor 4 (TLR4) (314). Furthermore TLRs have been expressed in healthy and allergic conjunctiva in VKC patients (315,316) and TLRs are also expressed *in vitro* by conjunctival epithelial cell cultures (317). TLR2 expression was up-regulated on conjunctival epithelial cells by IFN γ and *Staphylococcus aureus* which is a common pathogen colonising the conjunctiva in AKC (317). It has been investigated by means of immunohistochemistry and relative real-time polymerase chain reaction the expression of TLRs in VKC tissues compared to control tissues. TLR2, TLR4, and TLR9 were expressed in the conjunctival epithelium and stroma of normal and VKC subjects. In VKC tissues, TLR4 was upregulated, TLR9 was downregulated, and TLR2 was slightly decreased compared to control tissues. Both showed a more intense immunoreactivity in the stroma. TLR4 was localized on CD4⁺ lymphocytes, eosinophils, and mast cells. Lymphocytes and eosinophils, but not mast cells, expressed TLR9. The upregulation and downregulation of TLR expression in healthy conjunctiva and VKC suggest that there may be a role for TLRs in VKC (315). TLR3 ligand polyI:C induces TSLP expression *in vitro* by primary conjunctival cell cultures (318). Similar responses were also observed in bronchial epithelial cell cultures (319). Corneal epithelial cells treated with TLR3, -5, -6 ligands (TNF α /IL-1 β) secreted TSLP mRNA (320).

In the *in vitro* pilot data of this thesis, single IOBA-NHC cell treatments with polyI:C, zymosan and LPS induced production of all markers (EGFR, VGFR, CD44) but at lower levels compared to pro-inflammatory cytokines and PMA. Taken all the facts described above together it may be suggested that in VKC and AKC additional pathogen immune mediated mechanisms may run in parallel accounting for disease severity.

5.3 Classification of ocular allergic disease and novel therapeutic strategies

5.3.1 Re-classifying Ocular allergic disease

Both proposals for the grading of AKC and VKC recognised the absence of a helpful classification system based on symptomatology, clinical signs and requirement for decisions to treat these conditions. Further advances have been made since then that enhance our understanding about the immunopathology and pathogenesis which could assist in re-classifying the forms of the full spectrum of ocular allergy. An updated classification system could focus on proactive management suggestions based on available therapies. Therefore re-enforcing our understanding of the immunopathological processes could help to find a common consensus between ophthalmologists, allergologists and immunopathologists.

In 2011 ten Latin American ocular allergy experts participated in a 4-round Delphi panel approach aiming to define, classify, stage and provide diagnosis and treatment recommendations. It was concluded that the task of creating guidelines in ocular allergy was very complex and many controversial topics still remain unsolved. The Ocular Allergy Latin American consensus proposed that the term “ocular allergy” is the general term to describe ocular allergic diseases but a consensus on its classification and the role of systemic steroids was not reached. The panellists agreed on the need of a staging system based on disease severity and the need of a larger consensus with international groups of experts to improve recommendations on treatment (321).

A general consensus is required for Allergic conjunctivitis' classification and staging, based on clinical signs and decisions for therapy and there are significant difficulties to overcome when one attempts to classify ocular allergic diseases. AKC and VKC on some occasions may overlap yet AKC has no age limitation and involves the conjunctiva, the cornea and the lids. On the other hand if VKC is not “vernal” is frequently chronic. Many ophthalmologists recommend that if overlapping, the worse component must lead the management plan. The term “allergic conjunctivitis” may not sufficiently describe all forms of allergic eye disease, thus a new classification system is desirable, preferably derived from the varied pathophysiological mechanisms operating in the different forms of ocular allergy (322).

Finally, the recent Task Force report from the EAACI ocular allergy interest group on diagnosis and management of ocular allergy unifies the nomenclature and classification of ocular allergy, describes current methods to diagnosis and summarises the therapeutic options and management. The new classification proposed is shown below in Figure 5.3. The new classification is based on pathophysiology and hypersensitivity nomenclature and both IgE- and T cell mechanisms have been shown in VKC and AKC (323).

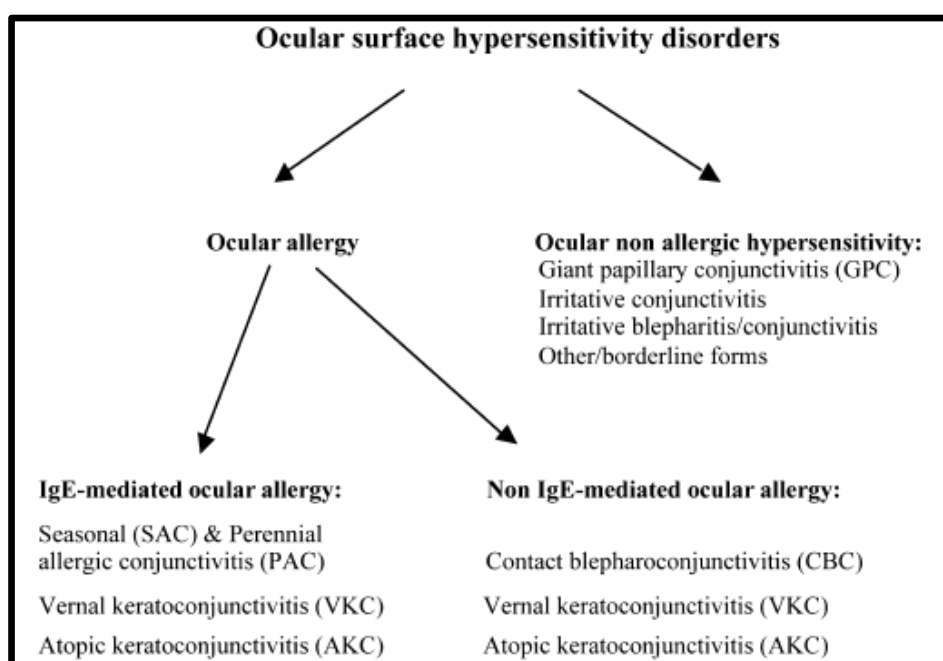


Figure 5.3: The New classification for ocular allergy proposed by the EAACI Task Force in 2012.

The IgE level of involvement has been used as the major feature when classifying ocular allergic diseases. Epithelial remodelling of the conjunctiva irreversibly alters the tissue function and structure. The results of this thesis suggest that epithelial remodelling co-participates in disease progression and perpetuation in CAED. It is not seen in reversible forms of disease without corneal involvement. Therefore it is suggested that it may be used as a pathologic feature to distinguish between the chronic and persistent (AKC, VKC) forms from the more acute and seasonal reversible types (SAC, PAC) (Table 5.2 below).

GPC shares features from the reversible forms of disease and some mild expression of remodelling molecules featuring in the severe forms AKC and VKC without permanent changes in cytological architecture (fibrosis, epithelial hypertrophy etc). Removal of the biomaterial reverses the clinical signs but its persistence leads to epithelial injury and cell activation leading to EGFR expression. The following table is proposed to present a summary of the immunopathological features of various form of ocular allergy

OCULAR ALLERGY	IgE Mediated	IgE & non-IgE mediated	Non-IgE mediated	Tissue Remodelling	Corneal involvement
Intermittent	SAC				
Persistent	PAC	VKC	GPC	VKC	VKC
Chronic		AKC	CDC	AKC	AKC

Table 5.2: A proposal for the features used for the classification of ocular allergy (Modified by reference 135)

5.3.2 New treatments, strategies and further studies

From a patient's perspective, symptom relief of the acute flare up episodes is the most desired aim of treatment with long term disease control. So far the instantaneous relief is not easy to achieve because of the irreversible nature of the chronic forms of ocular allergic disease. Most therapeutic strategies are centred on the inhibition of the allergic response and suppression of the inflammation. The long term complications of steroids make way for the use of steroid-sparing T cell immunomodulatory agents. It is clear that AKC and VKC require T-cell targeted strategies to achieve long term control. Figure 5.4 below summarises the current knowledge of the agents that can regulate the immune function at various levels in allergic disease. Immunity and inflammation have numerous features that overlap at the cellular and cytokine profile levels. In allergic conjunctivitis blocking CCR7 topically is shown to impair the pathogenic contribution of dendritic antigen presenting cells based on work carried out using CCR7 knockout mice C57BL/6 mice which is the only animal mouse model so far used in a study to investigate novel treatments in AED. CCR7 blockage leads to inhibition of Th2 reactivity and IgE synthesis as shown by this allergic conjunctivitis animal model (324).

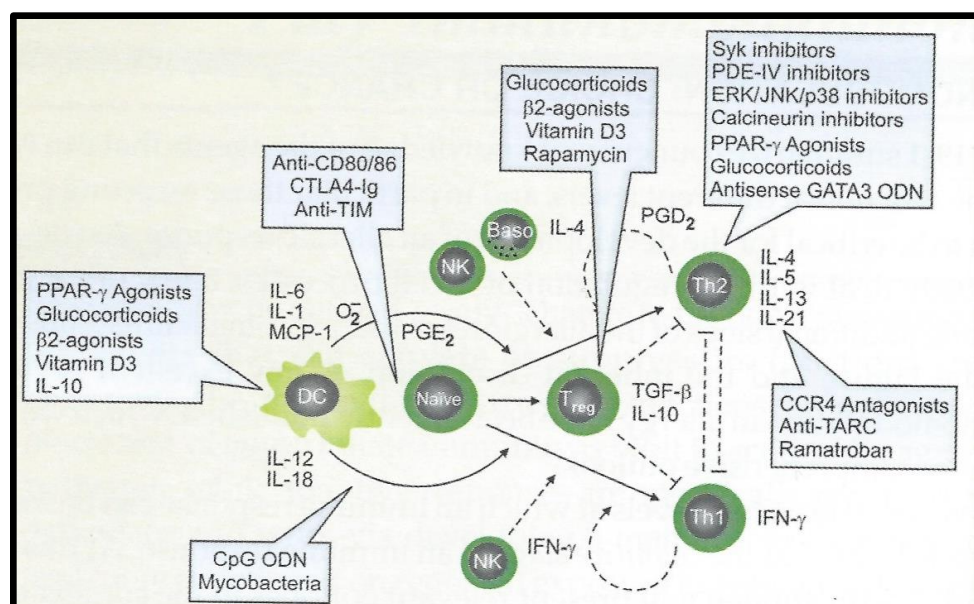


Figure 5.4: Multiple targets of immunomodulation in allergic disease (325).

Combinations of anti-allergic agents aim to inhibit histamine effects and induce an anti-inflammatory response. Levocabastine has antihistamine actions and reduces adhesion molecule expression (326). Additionally it reduces eosinophilia via increased expression of integrins in the conjunctiva (327). The efficacy when combining levocabastine with pemirolast is increased improving the benefits in SAC and PAC (328). Systemic administration of cyclosporine A and tarcolimus lead to complete remission in 6 patients with AKC (329) Administration of probiotic eye drops with *Lactobacillus acidophilus* in patients with mild and moderate VKC showed significant improvement after 4 weeks of treatment in six out of the seven patients (330).

The current treatments for asthma achieve little effect on the natural history of the disease and are based on disease severity and control rather than targeting the pathogenic mechanisms. The importance of subphenotyping asthma into different endotypes with different causal mechanisms has been described (331). Novel pathways causing disease that explain better the development and progression of asthma, may form a better basis for treatments that combat disease perpetuation rather than reduce an inflammatory response and temporarily induce symptom relief. It is important to identify those patients who would respond to biologic therapies targeting markers of Th2 dominant disease (331).

The results of this study raised a number of questions: would primary cell cultures from normal and allergic conjunctival tissues share similar functional responses to cytokines? In the future to address these issues, conjunctival biopsies need to be obtained from patients with different conjunctival inflammatory disease. Secondly, are the treatments currently available, have an effect on EMTU? It would be important to know if patients who have received early immunosuppression treatment develop evidence of EMTU activation and disease progression. Furthermore, based on the roles of EGFR and VEGF described in this study, what would be the effect of anti-VEGF and anti-EGFR agents on EMTU development and the cytokine profiles in the forms of ocular allergy?

5.3.3 EGFR inhibition as a possible new therapeutic strategy to control inflammation in chronic asthma and chronic allergic eye disease

Currently there are both *in vitro* and *in vivo* studies in animal regarding tyrosine kinase inhibitors and airways remodelling in asthma. EGFR inhibition has been shown to reduce epithelial cell proliferation, decrease collagen deposition and goblet cell proliferation in the airways (333). However the clinical application of these findings has not been translated so far due to the complex nature of the disease especially in severe cases. The role of anticancer drug gefitinib, an EGFR inhibitor was investigated, for the control of allergic airway inflammatory disease. More specifically, pre-treatment with gefitinib reduced IL-4 and IL-13 concentrations in bronchoalveolar lavage fluid as well as eosinophil recruitment in the lung in a dose dependent manner (332). This effect was associated with reduced EGFR expression and activation in mice. Therefore a similar role may be applicable in chronic allergic disease forms.

In fact EGFR expression was reported to be increased in an animal model of colitis (334) and EGF *pr* administration was beneficial in left sided ulcerative colitis (335) in which it was reported that there is serum EGF downregulation (336). This is of particular interest since it demonstrates the physiological mitogenic properties of EGFR ligands which promote epithelialisation and wound healing. On the contrary in chronic asthma and chronic allergic eye disease the EGFR expression and its ligands demonstrate abnormal epithelial repair and remodelling which are suggested to be accountable for the disease severity and steroid non-responsiveness. Therefore it could be hypothesised that EGFR inhibition in chronic mucosal inflammatory disease of allergic origin may be beneficial. It is therefore proposed that similar studies are designed to investigate the immunomodulatory role and effects on cytokine profiles and remodelling *in vitro* of anti-EGFR and anti-VEGF agents and *in vivo* in CAED.

This thesis showed by means of immunostaining using GPC biopsies from symptomatic patients that the EMTU remodelling epithelial molecules are lightly expressed. GPC as described in previous sections can be seen in contact lens wearers due to the biomaterials used. It is therefore proposed that when designing

contact lens materials, the EMTU molecules may be used as a screening tool to investigate wheather the biomatterial used is capable of inducing their upregulation in vivo using a CAED animal model as described above.

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APPENDIX: Patient details

GIANT PAPILLARY CONJUNCTIVITIS

NUMBER	SEX	ETHNIC GROUP	AGE	AGE AT ONSET OF DISEASE	SEASONAL VARIATIONS	HISTORY OF ATOPY	CURRENT TREATMENT
GPC 1	F	CAUC	26	20	APR-OCT (CONT)	AS, PR, HF, URT, AE, FA	NIL
GPC 2	F	W.INDIAN	28	16	AUG(CONT)	AS, PR, HF	DSCG
GPC 3	F	CAUC	40	28	APR-JUN (EPIS)	AS, PR, HF	NIL

VERNAL KERATOCONJUNCTIVITIS

NUMBER	SEX	ETHNIC GROUP	AGE	AGE AT ONSET OF DISEASE	SEASONAL VARIATIONS	HISTORY OF ATOPY	CURRENT TREATMENT
VKC 1	F	CAUC	25	3	NONE(CONT)	AS,PR,HF, URT,AE	DSCG,DEX
VKC2	F	CAUC	27	23	NONE(CONT)	AE	DSCG
VKC3	M	IND	18	13	NONE(CONT)	NONE	DSCG
VKC4	M	AFR	26	7	MAY-AUG	AS,PR	DSCG

ATOPIC KERATOCONJUNCTIVITIS

NUMBER	SEX	ETHNIC GROUP	AGE	AGE AT ONSET OF DISEASE	SEASONAL VARIATIONS	HISTORY OF ATOPY	CURRENT TREATMENT
AKC1	F	CAUC	29	1	NOV-JAN	AS,PR,HF,URT	NONE
AKC2	M	BARBADOS	59	55	NONE	AS,URT	DSCG,PRED
AKC3	F	CAUC	42	9	NONE	AS,PR,HF URT	DSCG,PRED
AKC4	F	IND	23	5	NONE	AS,PR,HF URT,FA	DSCG,PRED

CHRONIC OCP PATIENT DETAILS

Number	gender	age	DIF	IIF	Lubeck	Buccal bx	Topical Tx	Systemic Tx	Inflamm Grade R	Inflamm Grade L	TAUBER Upper/Lower	Foster stage R	Foster stage L	Allergies	Other eye pathologies	Eye biopsy	Extraocular pemphigoid
UP7	F	84	+ve CONJ	ND	NEG	ND	-	Mycophenol	1	0	IIb/IIcIIc(2)	3	3	-	-	L	-
UP9	F	66	LINEAF IgG IgA	ND	NEG	ND	-	Dapsone	0	0	IIb/IIcIIb(2)	3	3	-	-	R	-
UP13	F	76	Neg	NEG	IgA to LAD1	ND	Betoptic BD BE	Dapsone	1	1	IIc/IIcIIc(3)	3	3	-	glaucoma, entropion /trichiasis	L	-
UM2	M	59	linear IgA skir disease	NEG	NEG	ND	Celluvisc/Hypromel lacrilube	Cyclophos	1	1	R I/IIa, L I/IIa	2	2	AZA Ihd, MYCOPH bowel bleeding	-	L	SKIN
UM3	M	76	IgG IgA	NEG	NEG	IgA IgG	retinoic acid once/wk, acetyl/cysteine alt daily chlor qid, hypromellose	cyclophos 50mg/d	0.5	1.5	IIcIIId(2)/IIbIIb(2)	2	4	Vibramycin	Amblyopia, HSV	L	-
UM5	F	60	neg	NEG	NEG	ND	g pred 0.5% 2-4 times daily	Cyclophos 50mg alt daily, Dapso 100mg/d	1.5	1	R I/IIcIIc(3), L I/IIaIIa(1)	3	3	-	-	R	-

ACTIVE OCP PATIENT DETAILS

Number	gend	age	DIF	IIF	Lubeck	Buccal Bx	Topical Tx	Systemic Tx	Inflam Grade	Inflam Grade	TAUBER Upper/Lower	Foster si R	Foster S L	Allergies	Other eye pathologies	Eye biops	Extraocular pemphigoid
M1	F	54	IgG, IgA, C3, IgE conj & oral	IgG 1/10 roof	NC16A ELISA	POS	Celluvisc	nil	1	3	Ic1ld/ Ic1ld	3	3	nil	nil	R/L	mouth, nose, pharynx, larynx
M2	M	59	linear IgA skin disease	NEG	NEG	N/A	Hypromellose, lacrilube	nil	2.5	2.5	R I/ Ila, L I/ Ila	2	2	AZA, ihd, MYCOPH bowelbleeding	nil	R/L	skin
M6	M	55	IgG, IgA	NEG	NEG	POS	Dex once daily R, FML once daily L	Mycoph + dapsone + Deflazocort	3	2.5	R Ic1lc(2)/ Ic1ld(1), L Ib/ Ic1lb(1)	4	3	nil	RD BE, cataract	R/L	mouth
M7	M	74	NEG	NEG	IgG & IgA to LAD1	NEG	Trusopt, lumigan	Nil	2.5	2.5	R Ila/ Ic1lc(2), L Ila/ Ic1lc(2)	3	3	Preservatives	POAG	R/L	Nil
M9	M	81	IgG, IgA	IgG 1/10 roof	NEG	setterfield result pending	celluvisc	Mycoph + dapsone		2.5	Ila1la(1)/ Ib1lb(2)		3	Nil	POAG	L only	Nil
IP12	F	72	NEG	NEG	IgG to LAD1	N/A	Ofloxacin, Cyclopentolate, celluvisc	Nil	4	1	Ila/ Ic1lc(2)	3		nil	R pseudom keratitis	R	nil
IP14	M	72	nonspecific fibrin BMZ band	NEG	NEGN	NEG	viscotears, lacrilube, saline drops	10mg Pred for PMR, hydroxychloroquine	3	3		3	3	Nil	Schimer's 2mm R, 3mm L	R/L	Nil
IP6	F	64	positive vulval & mouth	Neg	N/A	POS	G. Pred bd RE	Nil	1		R Ila/ Ib1lc(2)	3		Nil	Schimer's 12mm BE	R	vulva, oral, skin-palms, bottom, nose